# PURIFICATION AND CHARACTERISATION OF PUTATIVE PROTEIN ALLERGENS FROM THE SEEDS OF THE CASTOR OIL PLANT, *RICINUS COMMUNIS*

by

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## Declaration

The data presented in this thesis are the results of original research conducted by the author under the supervision of Dr. J. M. Lord. Experiments involving Western blot analysis of castor bean proteins probed with human IgE were conducted in collaboration with Dr. D. M. Kemeny and Mrs. S. Thorpe, Department of Medicine, Guy's Hospital Medical School, London. All other experiments were conducted solely by the author.

None of the data presented in this thesis have been used in a previous application for a degree.

## Abbreviations

| | |
|---|---|
| A | absorbance at the wavelength indicated as a subscript. |
| Abstr. | abstract. |
| AgE | ragweed antigen E. |
| AgK | ragweed antigen K. |
| BAEE | αN-benzoyl-L-arginine ethyl ester. |
| BBI | Bowman-Birk inhibitor. |
| Bp | *Bordetella pertussis* vaccine. |
| BSA | bovine serum albumin. |
| BTEE | αN-benzoyl-L-tyrosine ethyl ester. |
| C | centigrade. |
| CB-1A | a highly allergenic protein extract from castor beans. |
| CB pool | pooled sera from ten patients known to give a positive skin prick reaction when tested with castor bean extract. |
| cDNA | complementary DNA. |
| CFA | Complete Freund's Adjuvant. |
| CM | carboxymethyl. |
| Co. | company. |
| CPM | counts per minute. |
| CRD | cross-reactive doublet. |
| CRIE | crossed radioimmunoelectrophoresis. |
| DNA | deoxyribonucleic acid. |
| DNAse | deoxyribonuclease. |
| dNTP | deoxynucleoside triphosphate (A = adenosine, C = cytosine, G = guanosine and T = thymidine). |
| DPM | disintegrations per minute. |
| DTT | dithiothreitol. |
| E. coli | *Escherichia coli*. |

| | |
|---|---|
| EDTA | ethylenediamine tetraacetic acid. |
| ELISA | enzyme-linked immunoabsorbent assay. |
| FcR | receptor for the constant region of an immunoglobin. |
| g | gramme. |
| h | hour. |
| HEPES | N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid. |
| HLA | human MHC. |
| I region | region of the MHC to which Ir genes map. |
| Ig | immunoglobulin. |
| Ir gene | immune response gene. |
| $K_a$ | affinity constant, applied specifically in this thesis to the binding of IgE to its receptor. |
| kb | kilobase. |
| kDa | kilodalton, a unit of mass. |
| KLH | keyhole limpet haemocyanin. |
| L | litre. |
| Ltd. | limited. |
| LTI | lima bean trypsin inhibitor. |
| Lyt | cell-surface antigen used to distinguish between populations of T-cells. |
| M | molar. |
| 2-ME | 2-mercaptoethanol. |
| mg | milligrammes. |
| MHC | major histocompatibility complex. |
| min | minute. |
| mRNA | messenger RNA. |
| ng | nanogramme. |
| nm | nanometre. |
| NMR | nuclear magnetic resonance. |

| | |
|---|---|
| P1 | amino acid residue in the reactive site of serine protease inhibitors which determines the specificity of the inhibitor. |
| PAS-staining | periodic acid-schiff staining. |
| PBS | phosphate buffered saline. |
| PCMB | p-chloromercuribenzoate. |
| pg | picogramme. |
| PMSF | phenylmethylsulphonylfluoride. |
| pI | isoelectric point. |
| PSI | pounds per square inch. |
| Rd. | road. |
| RNAse | ribonuclease. |
| RNAsein | ribonuclease inhibitor. |
| RPM | revolutions per minute. |
| S | Svedberg, sedimentation coefficients are expressed in Svedberg units, 1S = $10^{-13}$ sec. |
| SDS | sodium dodecyl sulphate. |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis. |
| TCA | trichloroacetic acid. |
| TEMED | N,N,N,N'tetramethylenediamine. |
| Tris | tris (hydroxmethyl) aminomethane. |
| var | variety. |
| v/v | volume to volume. |
| w/v | weight to volume. |
| Amino acids | standard three letter codes are used to indicate both free amino acids and amino acyl residues comprising proteins. |

## Summary

The identification, purification and characterisation of putative castor bean allergens has been achieved together with some studies on the synthesis of castor bean cDNA undertaken as a prerequisite to the isolation of allergen cDNA clones.

At least four proteins have been identified which bind immunoglobulin E from the pooled sera of ten patients allergic to castor beans (CB pool). The apparent molecular weights of these proteins are 10,000, 34,000, 48,000 and 50,000. On the basis of their ability to elicit an IgE response these proteins are considered to be putative castor bean allergens.

The 10 kDa putative allergen has been identified as the large subunit of a heterodimeric 2S storage albumin purified in the course of this study. The putative 2S allergen has been shown to be identical to a castor bean 2S albumin previously purified and sequenced by Li and coworkers (Li et al., 1977; Sharief and Li, 1982). In consequence, the putative 2S allergen has been called Li's protein.

Li's protein has been shown to be a trypsin inhibitor. Li's protein may be encoded by a multigene family and is initially translated as a 32.5 kDa precursor, almost three times the mass of the mature protein (11 kDa) as determined by amino acid sequencing.

IgE from the CB pool specifically binds to two components of the complex crystalloid group of storage proteins. The 34 kDa subunits of these crystalloid proteins may be the putative 34 kDa allergen. The binding of IgE from the CB pool by components of the crystalloid complex may be the result of a cross-reaction with IgE raised against the 2S allergen.

The putative crystalloid allergens have been substantially purified by ion-exchange chromatography. The feasibility of using chromatofocusing to rigorously purify the putative crystalloid allergens and to separate the individual polypeptides has been investigated.

The S1 nuclease method was found to be preferable to the RNAse H method for the synthesis of castor bean cDNA.

The 48 kDa and 50 kDa putative castor bean allergens have not been identified.

# CHAPTER 1

# INTRODUCTION

# SECTION 1

# AIMS AND DEFINITIONS

The aim of this thesis is to describe the isolation and characterisation of putative protein allergens from the seeds of the castor oil plant, Ricinus communis.

To appreciate both the background to this work and the potential applications of the results in immunotherapy some knowledge is required of a range of diverse subjects including; cellular immunology, the properties of protein allergens and the characteristics of the three classes of storage proteins found in castor beans. It is the aim of the Introduction to provide this information as five broad subject areas each presented as a separate section.

The first section explains the structure of the Introduction and provides a short glossary of terms which are basic to the arguments and results presented in this thesis. The second section describes the clinical symptoms of the type of allergic disease caused by exposure to castor bean dust and the role of immunoglobulin E. The third section describes the properties of selected groups of allergens, including the castor bean allergens, and summarises what is known of the general properties of allergens. The fourth section describes the three classes of storage proteins found in castor beans since previous work implicated at least one class of storage proteins, the 2S albumins, as castor bean allergens (see Sub-section I 4(D)). The final section of the Introduction summarises the wealth of information concerning serine protease inhibitors found in plants since evidence is presented in Section 5 of the Results and Discussion Chapter that an important, putative castor bean allergen is a trypsin inhibitor.

The discussion of the castor bean storage proteins and plant serine protease inhibitors together with the summary of the previous work on the castor bean allergens are of greatest relevance to the results

presented in this thesis. The discussion of the general properties of allergens and a limited discussion of cellular immunology mainly serve to provide the conceptual background to the brief review of current approaches to immunotherapy, particularly those utilising characterised allergens, which is presented in the Concluding Chapter of this thesis.

Those terms which are judged to be basic to the arguments and results presented in this thesis are explained and defined below:

Allergy: the term "allergy" was introduced by von Pirquet (1906) to describe all states of altered immunological reactivity in animals. In its original form it includes both protective immunity and hypersensitive responses resulting from immune reactions which cause tissue damage (Coombs and Gell, 1975). In this thesis the term allergy will be restricted in its application to a hypersensitive reaction of the immediate (Type 1) group according to the classification of Coombs and Gell (1975) described below.

Immediate Hypersensitivity: Coombs and Gell (1975) developed a classification scheme for allergic reactions responsible for clinical hypersensitivity and disease. Their classification was primarily based on the initiating mechanism of the allergic response. According to their scheme allergic reactions producing local or systemic damage and operative in clinical hypersensitivity could be divided into four groups: Type 1, immediate; Type 2, cytotoxic; Type 3, damage by antigen-antibody complexes and Type 4, delayed or cell-mediated. Of these four only Type 1 is of relevance to this thesis since this type of allergic reaction may be elicited on repeated exposure to castor bean dust (Ordman, 1955). Following the definition of Coombs and Gell a Type 1 reaction is "initiated by allergen or antigen reacting with tissue cells (basophils and mast cells) passively sensitised (allergised) by

antibody produced elsewhere, leading to the release of pharmacologically active substances."

Antigen: a substance which stimulates the production of antibody (Playfair, 1984).

Antigenic Determinant: a region of a protein antigen that is recognised by antibodies or T-cells when the whole protein is the antigen (after Atassi, 1984).

Allergen: antigens that induce immediate or delayed hypersensitivity in man are termed allergens (King, 1976). This thesis is concerned only with allergens causing immediate hypersensitivity such as those found in castor beans. Most known allergens are proteins (King, 1976) therefore the term "allergen" will indicate a protein allergen unless otherwise stated.

# SECTION 2

# IMMEDIATE HYPERSENSITIVITY AND THE ROLE OF IgE

## Immediate Hypersensitivity

Repeated exposure to castor bean dust may elicit a Type 1 hypersensitivity reaction (Ordman, 1955). The clinical symptoms of a Type 1 reaction vary depending on the mode of challenge with the allergen. The symptoms of a generalised Type 1 reaction include; bronchial asthma, pulmonary oedema and urticaria (rash). In a localised Type 1 reaction, such as may be elicited by a skin-prick test (intracutaneous injection of a small dose of allergen) the symptoms will include local oedema and inflammation (Coombs and Gell, 1975). It is important to note that a subject showing a local Type 1 reaction is generally sensitised to the allergen.

A Type 1 hypersensitivity reaction depends on the production of antibodies, originally termed reaginic antibodies, against a specific allergen or group of allergens. Ishizaka and colleagues (Ishizaka et al., 1966; Ishizaka and Ishizaka, 1968) demonstrated that immunoglobulin E (IgE) was the principal carrier of reaginic antibody activity. IgE is produced in lymphoid tissue associated with the gut and upper respiratory tract (Callerame et al., 1971; Tada and Ishizaka, 1970) and is transported into the blood where it becomes associated with cells bearing IgE receptors, principally tissue mast cells and circulating basophils (Ishizaka, 1973). These target cells are described as being passively sensitised to the allergen whereas the B-cells producing antibody are actively sensitised. On subsequent exposure to the allergen the passively sensitised mast cells and basophils release a range of pharmacologically active substances, the general effects of which are to cause local oedema, swelling due to the collection of fluid in the tissue, and smooth muscle contraction (Coombs and Gell, 1975).

The chemical mediators released by mast cells and basophils include: histamine, slow-reacting substance of anaphylaxis (SRS-A) and an eosinophil chemotactic factor (ECF-A), (Goetzl and Austen, 1980; Kay and Anwar, 1980).

The presence of ECF-A and other eosinophil chemotactic factors is important in the regulation of the hypersensitive response since eosinophils attracted by the ECF-A can degrade vasoactive substances released by mast cells; thus histaminase released by eosinophils degrades histamine and arylsulphatase B inactivates SRS-A. By ensuring a local accumulation of eosinophils and platelets, through mast cell secretion of the appropriate chemotactic factors, a local inflammatory response occurs since these cell types are stimulated to release inflammatory mediators by the binding of IgE to receptors on their surface (Capron et al., 1986).

## Structure and Biological Activity of IgE

The antibodies responsible for immediate hypersensitivity have been called homocytotropic or reaginic antibodies (Coca and Cooke, 1923; Roitt, 1980). Ishizaka and Ishizaka (1967) first demonstrated that reaginic antibodies in the serum of hay-fever sufferers were of the IgE class. The identification of an IgE myeloma protein allowed detailed characterisation of this antibody class (Bennich et al., 1969). In common with other immunoglobulin classes IgE is composed of four polypeptides, two light chains and two heavy chains. It is the heavy chains which carry the isotypic determinants, the structural features distinguishing IgE from other classes of immunoglobulins (Geha, 1984). Both IgE and IgM have five structural domains stabilised by intra-chain disulphide bonds, in contrast to the other immunoglobulin classes which have only four domains. The extra domain is located at the carboxyl terminus of the heavy chain and mediates the interaction between IgE and its target cells (Ishizaka et al., 1970). Of the five classes of immunoglobulins IgE is the least abundant, having a mean serum concentration in non-allergic individuals of between 17 and 450 ng/ml which represents only 0.002% of total immunoglobulin (Roitt, 1980).

The most important biological property of IgE is the ability to bind and sensitise homologous tissue. That IgE is the sole reaginic class of antibody was demonstrated by Ishizaka and Ishizaka (1975). They showed that intracutaneous injection of $10^{-4}$ $\mu$g of antibody specific for IgE (anti-IgE) resulted in an inflamed wheal. Injection of even a thousand-fold greater amount of antibodies specific for the other immunoglobulin classes did not provoke a reaction. In this experiment the anti-IgE antibodies acted like an antigen in binding the small

amount of immobilised IgE found in normal sera and tissue. In addition, it was shown that monkey and human lung tissue could be passively sensitised in vitro with serum containing IgE directed against antigen E, an allergen from the ragweed plant, Ambrosia elatior. Exposure to antigen E resulted in the release of vasoactive amines and constriction of smooth muscle. The sensitising ability of the serum could be removed by prior absorption with anti-IgE (Orange et al., 1971).

The cells to which IgE binds are tissue mast cells and basophils since only these bind $^{125}$I-labelled anti-IgE (Geha, 1984). Binding of IgE to its target cells occurs by means of an interaction between the carboxyl terminal domain of the IgE heavy chain and a specific cellular receptor (Ishizaka et al., 1970). The solubilised receptor from mast cells and basophils is monovalent with respect to the binding of IgE (Conrad and Froese, 1976). Under physiological conditions the affinity of IgE for basophils is very high; the equilibrium constant ($k_a$) for the binding between IgE and receptor is of the order of $10^{-9}$M (Ishizaka et al., 1973). The binding is not, however, covalent since cell-bound IgE dissociates when exposed to a pH less than 4 (Ishizaka and Ishizaka, 1974). The strong affinity of IgE for receptors on target cells explains why a minute dose of IgE can cause persistent sensitisation. Considerable effort has recently been focused on elucidating the structure of this class of high-affinity IgE receptor (Metzger et al., 1986).

Mast cells and basophils are the principal groups of cells involved in a Type 1 hypersensitivity response. As mentioned in Sub-section I 2(A), though, other cell types bear specific IgE receptors, notably macrophages, eosinophils and platelets (Spiegelberg, 1984; Capron et al., 1984; Joseph et al., 1983). This type of receptor is distinct from that found on mast cells and basophils, having a $k_a$ of about $10^{-7}$M for monomeric IgE and $10^{-8}$M for dimeric IgE (Metzger et al., 1983). The

higher $k_a$ for the interaction with dimeric IgE may be functionally important since IgE complexes are known to be formed in allergic disease (Brostoff et al., 1977). More importantly macrophages, eosinophils and platelets from allergic individuals bear IgE antibodies on their surfaces (Joseph et al., 1981, 1983; Capron et al., 1985). Activation of these sensitised cells by the appropriate allergen releases an array of inflammatory mediators both in vitro and in vivo (Joseph et al., 1981). It is likely, then, that these cell types are specifically activated during a Type 1 response, a more specialised role than their known contribution to general inflammatory responses (Capron et al., 1986).

The binding of antigen to cell-bound IgE is not, itself, sufficient to stimulate the release of vasoactive amines since monovalent antigens are unable to induce an allergic response (Levine and Redmond, 1968). This observation suggests that cross-linking of IgE is important in stimulating target cells. The indirect bridging and aggregation of IgE receptors by multivalent antigen bound to immobilised IgE may, in turn, be the crucial initiating step in a Type 1 response. This idea was tested in non-allergic rats by direct bridging of the IgE receptors using a monoclonal antibody raised against a rat IgE receptor (Ishizaka et al., 1977). The anti-receptor antibodies induced histamine release from unsensitised mast cells, suggesting that aggregation of receptors is an important initial step in activating cells involved in the IgE response.

The existence of the IgE class of antibodies despite the deleterious hypersensitivity reactions associated with it suggests that this class of antibodies may serve an important function in adaptive immunity. The most convincing explanation for the normal role of IgE is that it has evolved as a defence against helminthic parasites (Platts-Mills, 1975). Dessein et al. (1981) found that IgE-deficient rats had

two to three times more Trichinella larvae encysted in their muscle than did control litter mates. In addition, IgE-dependant cellular cytotoxicity involving macrophages, eosinophils and platelets has been demonstrated using parasitic schistosomal worms (Capron et al., 1975; Joseph et al., 1980). In this context there is an obvious role for mast cells and basophils which release a range of pharmacologically active mediators on stimulation, notably eosinophil chemotactic factors and a platelet aggregating factor (see Sub-section I 1(A)). Mast cells and basophils may, then, act as "gatekeepers" against helminthic infection (Capron, 1986). It is noteworthy that rare individuals with no detectable IgE are apparently healthy, suggesting that IgE does not play a vital role in the normal physiology of the organism.

# SECTION 3

# THE CHARACTERISTICS OF PROTEIN ALLERGENS

## The Characteristics of Some Clinically Important Allergens

A large and increasing number of allergens have been identified and characterised. The large number of known allergens renders an exhaustive description of each prohibitive. Instead, two groups of clinically important and extensively characterised allergens will be discussed in detail; the ragweed pollen allergens and the honeybee venom allergens. The limited available data on the properties and identity of the castor bean allergens will also be presented. From these examples and with reference to other allergens where necessary the distinguishing characteristics of protein allergens will be described. For an indication of the large number and broad range of known allergens the reader is referred to the most recent reviews in this area by Marsh (1975) and King (1976).

## Ragweed Pollen Allergens

The ragweed pollen allergens are undoubtedly the best characterised group of allergens and have been subject of numerous studies. The pollen of ragweed, a member of the Compositae family (Wodehouse, 1971), is the principal cause of late-summer hayfever in the eastern United States and Canada (King, 1976). The two most abundant species of ragweed in these areas are Ambrosia trifida and Ambrosia elatior, known as tall and short ragweed, respectively. Although most work has concentrated on the pollen allergens of A. elatior they have been shown to be antigenically related to A. trifida by immunodiffusion studies with separate pools of rabbit antisera raised against both groups of proteins (Wodehouse, 1954; Goldfarb et al., 1958).

There are at least 14 antigens in an aqueous extract of short ragweed pollen (Gussoni, 1966). Of these the two major allergens, designated antigens E (AgE) and K (AgK) are unglycosylated, acidic proteins with molecular weights of about 38,000 and isoelectric points (pIs) in the range 4.8 to 6.0 (King et al., 1964; King, 1972). Antigens E and K represent 6% and 3% of pollen proteins, respectively. Antigen E consists of two major and two minor isoallergenic variants (King et al., 1964). As the name implies isoallergenic variants are closely related proteins which differ slightly in their physicochemical properties but which are allergenically indistinguishable (Roebber et al., 1982).

AgE is a heterodimer composed of two polypeptide chains of molecular weights about 26,000 and 13,000 (Marsh, 1975) which associate non-covalently. The two chains differ in both molecular weights and amino acid composition. Urea-denatured AgE was shown to be about ten thousand-fold less allergenic than the native AgE when assayed by skin-prick tests on sensitive individuals. Neither did the denatured AgE

react with sheep anti-AgE antibodies raised against native AgE (King, 1976). Reduction of the three disulphide bonds in AgE and carboxymethylation of the free thiol groups resulted in an AgE derivative which had less then 0.0001% of the allergenicity of the native antigen as shown by skin-prick tests (King et al., 1967b). Similarly, extensive chemical modification of AgE by acetylation, succinylation or butyrimidation of its amino groups or coupling of its carboxyl groups with taurine or glycinamide abolished the biological activity of the protein (King et al., 1967b, 1974). Compared to the native antigen the denatured antigen is a poor immunogen in both rabbits and mice and the antisera produced cross-react poorly with the native antigen (King et al., 1974).

Three other ragweed allergens have been purified and designated Ra3 (Underdown and Goodfriend, 1969), Ra4 (Griffiths and Brunet, 1971; Griffiths, 1972; Roebber, 1975) and Ra5 (Lapkoff and Goodfriend, 1975; Roebber et al., 1975). They are all basic proteins with molecular weights of 11,000, 23,000 and 4,970, respectively. They represent, respectively, 0.4%, 0.1% and 0.9% of the pollen proteins. Ra3 is the only ragweed allergen known to be glycosylated (Roebber and Goodfriend, 1970). The complete amino acid sequence for Ra5 is known (Mole et al., 1975). Two isoallergens of Ra5 have been identified, Ra5A and Ra5B (Roebber et al., 1982). Sequencing of the first 30 amino acids of both revealed complete sequence homology to each other suggesting that the differences between the two reside near the carboxyl terminus. Although IgG antibodies from hyperimmunised rabbits could not distinguish between the two forms both human IgG and IgE could distinguish between the two forms in 10-20% of the cases using serum from sensitised subjects. The existence of different forms of an allergen, immunologically indistinguishable by sera from most hypersensitive subjects provides a potentially useful tool for the identification of sites not contributing

to the allergenicity of the protein (Roebber et al., 1982). More recently, a homologue of Ra5, Ra5G, has been purified from giant ragweed (Roebber et al., 1985). It has a slightly lower molecular weight than Ra5, 4,000 compared to 4,970, and also has a lower pI, 8.3 as compared to 9.6 for Ra5.

The five ragweed allergens do not entirely account for the allergenicity of short ragweed pollen. Goldfarb (1968) isolated 16 partially purified allergenic fractions by aqueous extraction of ragweed pollen. Three of these fractions contained little AgE but were more allergenic than fractions rich in AgE. More recently Hussain and Marsh (1979) identified a rapidly extracted, basic ragweed allergen designated Ra6.

Direct skin testing on ragweed sensitive individuals (King et al., 1964) and histamine release assays with leucocytes from sensitised donors (Lichtenstein et al., 1966) showed that AgE was the most active ragweed allergen. Direct skin tests on 184 sensitive individuals showed that the median minimal amount of AgE required to elicit a positive skin test reaction was about 1 pg. A number of comparative studies on the allergenic activities of Ra3, Ra4 and Ra5 with AgE have been conducted (Lichtenstein et al., 1973; Santilli et al., 1975; Marsh et al., 1973). The results show that Ra3 and Ra5 are highly allergenic in 20-30% of sensitive individuals as compared to the high activity of AgE in 95% of the subjects.

By studying the kinetics of release, in vitro, of the known ragweed allergens Marsh et al. (1981) cast doubt on the status of AgE as the major ragweed allergen. They found that AgE was released slowly during aqueous extraction compared to the other allergens; Ra5, for example, had been completely released within four minutes. Previous work by Bridger and Proctor (1971) had shown that $^{99}$Tc-labelled albumin spheres 15 to 25 $\mu$m in diameter were cleared from nasal mucosa in six to eight

min. The spheres were deposited in the larynx where they remained for about 20 min. and were then swallowed. These results were considered to be indicative of the fate of inhaled ragweed pollen grains which are of approximately the same diameter as the albumin spheres, 18 μm. (Marsh et al., 1981).

It is likely, therefore, that AgE is not the major allergen causing the rapid, initial reaction to ragweed pollen, although the earlier work of King et al. (1964) showed that it is still a major allergen in eliciting the overall response. They demonstrated that the allergenic activity of a long-term aqueous extract of ragweed pollen was reduced 10-fold by removal of AgE with specific rabbit anti-AgE antibodies. Further, Fabricaint (1941) had shown that the normal pH range of the nasal mucosa was pH 5.5 to 6.5, the conditions under which the extraction experiments described above were performed. Under conditions of allergic rhinitis, however, the range of pH rises to pH 7.2-pH 8.3. At the higher pH range AgE is extracted ten times faster than at the lower pH range. Thus, allergic rhinitis caused by ragweed allergens other than AgE would progressively favour the release of AgE. This work also supports the idea that there are other, as yet unidentified, allergens in ragweed pollen since the allergenicity of brief aqueous extracts could not solely be accounted for by the minor allergens to which only about 13% of the ragweed-sensitive subjects reacted.

Marsh (1975) estimated the mean annual doses of three ragweed allergens, AgE, Ra3 and Ra5, in the Baltimore area of the United States and determined values of 0.9, 0.15 and 0.06 μg, respectively. These values are undoubtedly overestimates as a 24 hour per day exposure period was assumed as well as the complete extraction of the allergens. The latter assumption is clearly invalid in view of the subsequent work on the kinetics of release of ragweed pollen allergens described above. These results emphasize the extremely small doses of allergen required

to elicit a hypersensitive response.

The most recent work on the ragweed allergens has concentrated on the elucidation of the three-dimensional structure of Ra5 and identification of structural features important to its antigenicity and, hence, allergenicity. Galley et al. (1982) investigated the phosphorescence and fluorescence emission properties of two Ra5 tryptophan residues with a view to using them as intrinsic probes with which to monitor the effects of specific structural modifications of the protein. Subsequent $^{1}$H-nuclear magnetic resonance studies by Vidusek et al. (1985) allowed assignment of the four disulphide bonds of Ra5 which, together with secondary structure predictions, enabled a solution structure for Ra5 to be generated. Features of the predicted structure centred around four disulphide bonds are found in other proteins, including wheat germ agglutinin and hevein (Drenth et al., 1980).

An important technical advance has been the solid phase synthesis of the complete Ra5 molecule composed of 45 amino acids (Choudhury and Goodfriend, 1983). The protein was allowed to fold and form disulphide bonds spontaneously. The synthetic Ra5 was shown to be very similar in conformation to the native protein by a variety of techniques including circular dichroism and immunoassays although the allergenicity of the protein was not tested. It is now possible to synthesise specifically modified derivatives of Ra5 useful in the study of the genetic and cellular control of the IgE response to this clinically important allergen.

## Honeybee Venom Allergens

The venom components of honeybee, Apis mellifera, have been extensively characterised by Habermann (1972). The identified components include a hyaluronidase, a phospholipase $A_2$, a haemolytic peptide (melittin) and a neurotoxic peptide, apamin. Their respective molecular weights are 50,000, 15,800, 2,840 and 2,000 (King, 1976). The complete amino acid sequence and disulphide bond assignments are known for phospholipase $A_2$ (Shipolini et al., 1974a and 1974b). In addition Habermann described a 22 amino acid peptide which mediates the non-cytolytic degranulation of mast cells (Haberman, 1972). This mode of action is in contrast to that of melittin and phospholipase which cause the release of vasoactive amines from mast cells by cytolysis. Two other allergens were subsequently isolated and identified as an acid phosphatase (Hoffman, 1977) and an α-glucosidase (Shkenderov, 1979). Bee venom also contains three pharmacologically active amines; histamine, dopamine and noradrenaline (Haberman, 1972).

The allergenic activities of four of the protein venom components were tested by the histamine release assay; for most sensitised individuals phospholipase $A_2$ was most allergenic followed by hyaluronidase then melittin, apamin was not allergenic (Sobotka et al., 1976; King et al., 1976). In contrast, by measuring the amount of IgE bound to purified bee venom allergens using the radioallergosorbent technique (RAST), Arbesman et al. (1976) and Light et al. (1976) suggested that hyaluronidase may be of greater clinical importance than phospholipase $A_2$. Kemeny et al. (1983a) subsequently demonstrated that phospholipase $A_2$ is the major bee venom allergen, the antigen to which most patients (91%) made IgE antibodies and against which the highest

levels were most frequently measured (54% of allergic individuals). Important allergens in a minority of patients were hyaluronidase (17%), acid phosphatase (17%) and melittin (6%). The important feature of the study by Kemeny and colleagues was their use of RAST-inhibition to assess the specificity of the IgE response against individual, purified allergens. In general, RAST-inhibition involves incubation of serum from allergic patients with individually purified allergens from either the same source as the allergen of interest or from an unrelated source. The ability of the coexistent or extraneous allergens to inhibit the RAST-binding of IgE to the allergen of interest is then measured.

Bee venom phospholipase $A_2$ was chemically modified in experiments similar to those of King et al. (1967b and 1974) on ragweed AgE. The modifications included succinylation of the amino groups, cyanogen bromide cleavage of methionyl peptide bonds and reduction and carboxymethylation of the four disulphide bonds. All the modifications lowered the allergenicity of the protein, particularly reduction and carboxymethylation which resulted in a derivative with only $10^{-4}$ of the activity of the native protein (King et al., 1976). These experiments again illustrate the importance of charge and conformational integrity to the biological activity of allergens.

Melittin has a very low molecular weight but can associate to form an aggregate of 12,000 (Haberman, 1972). Apamin with a similar molecular weight of 2,000 cannot associate and, unlike melittin, is not allergenic. The property of self-association may therefore be important to the allergenicity of small allergens (Stanworth, 1973; King, 1976).

## The Castor Bean Allergens

Castor beans yield an unsaturated oil which has many industrial uses including the production of cosmetics, plastics and explosives (Panzani and Layton, 1963). The oil is produced by mechanical expression of the beans or by solvent extraction yielding a dry pomace which may be used as fertiliser (Berrens, 1971). Castor oil is not allergenic but the dust from the process can cause severe hypersensitivity in those exposed to it (Figley and Elrod, 1928; Ordman, 1955). Castor beans are unusual in that they can sensitize individuals not predisposed to allergy (Thorpe et al., in press).

The earliest attempts to isolate the castor bean allergens date back to the work of Spies and Coulson in the 1940s. They developed a protocol for the isolation of a highly allergenic castor bean fraction designated CB-1A (Spies and Coulson, 1943). Their protocol was based on the differential precipitation in ethanol and basic lead acetate of components of an aqueous extract of castor bean proteins. Although CB-1A was highly allergenic and formed the basis of many subsequent studies on the castor bean allergens (for a review see Berrens, 1971) it should be noted that the allergenic properties of castor bean components other than CB-1A were not tested. On the basis of immunodiffusion studies with serum raised against CB-1A Spies (1968) concluded that the CB-1A fraction contained at least eight distinct, potentially allergenic proteins. A sedimentation coefficient for a subfraction of CB-1A, CB-1A(E) which is the retained fraction of CB-1A which has been dialysed for 595 hours against water, was determined as 1.98 (Spies and Coulson, 1964).

CB-1A is extremely resistant to denaturation by extremes of heat and pH (Spies et al. 1962). Heating for one hour at 110° at pH 5.9 had no effect on the immunoprecipitating activity of CB-1A(E). Heating for one hour at 150°C at pH 5.9 was required to completely destroy the precipitating activity of CB-1A(E). This fraction also showed considerable stability to heating in alkaline pH. At pH 12, heating at 100°C and 120°C took 32 and 8 minutes, respectively, to completely destroy the allergenic activity as determined by means of a skin-prick test.

More recent work by Youle and Huang (1978b) compared the physicochemical properties of the CB-1A fraction with the castor bean 2S albumins, small, water-soluble storage proteins (Sub-section I 4(D)). They demonstrated that CB-1A and the 2S albumin fraction had similar electrophoretic mobilities on SDS-PAGE, a significant proportion of the 2S albumins were precipitated using the CB-1A isolation procedure of Spies and Coulson (1943) and antibodies raised against CB-1A precipitated the 2S albumins. It was concluded that the CB-1A fraction was composed, at least in part, of the 2S albumins.

A single component of the 2S albumin fraction has been purified (Li et al., 1977) and sequenced (Sharief and Li, 1982). The allergenic properties of the protein were not tested but sequence homology with the Bowman-Birk trypsin inhibitor of lima bean (Tan and Stevens, 1971), including the active site residues, suggested that it may be a trypsin inhibitor. The primary sequence of the purified 2S albumin is shown in Figure I1.

Figure I 1 The amino acid sequence of a castor bean 2S albumin

A castor bean 2S albumin was isolated by Li et al. (1977) and sequenced by Sharief and Li (1982) who demonstrated partial sequence homology between the large subunit of the castor bean albumin and a Bowman-Birk protease inhibitor isolated from lima bean (Tan and Stevens, 1971). The homologous region included the active site of trypsin inhibition. The sequence of the castor bean 2S albumin is presented in Figure I 1 and the proposed site of trypsin inhibition underlined.

Small Subunit

```
                                                    10
Pro-Ser-Gln-Gln-Gly-Cys-Arg-Gly-Gln-Ile-Gln-Glu-Gln-Gln-Asn-
                          20                                30
Leu-Arg-Gln-Cys-Gln-Glu-Tyr-Ile-Lys-Gln-Gln-Val-Ser-Gly-Gln-
                  34
Gly-Pro-Arg-Arg
```

Large Subunit

```
                                                    10
Gln-Glu-Arg-Ser-Leu-Arg-Gly-Cys-Cys-Asp-His-Leu-Lys-Gln-Met-
                          20                                30
Gln-Ser-Gln-Cys-Arg-Cys-Glu-Gly-Leu-Arg-Gln-Ala-Ile-Gln-Gln-
                                                  40
Gln-Gln-Leu-Gln-Gly-Gln-Asn-Val-Phe-Glu-Ala-Phe-Arg-Thr-Ala-
          Ser
                          50                                    61
Ala-Asn-Leu-Pro-Ser-Met-Cys-Gly-Val-Ser-Pro-Thr-Gln-Cys-Arg-Phe
```

## The General Properties of Protein Allergens

Comparison of a large number of protein allergens reveals some physicochemical characteristics common to most of them. Barren (1971) tabulated the sedimentation coefficients of 19 allergens and found that they all fall within the 2-4S range, corresponding to molecular weights of 25,000-40,000. Marsh (1975) noted that there was no known allergen of molecular weight greater than 60,000. It should be noted that molecular weight values usually cited are for allergens under denaturing conditions and give no indication of the ability of an allergen to self-associate.

Low molecular weight is generally regarded as a physical constraint imposed on allergens if they are to efficiently traverse the mucous membranes. In this respect the upper size limit of protein allergens corresponds to the upper size limit for permeation of the mucosal membranes at between 40 and 60 kDa (Schneeberger, 1974). In principle, there must be a lower limit on the possible molecular weight of an allergen since there is a requirement for sufficient structural complexity to provide a large array of B-cell determinants. This requirement is important if an allergen is to efficiently associate with B-cells of the appropriate specificity or cross-link immobilised IgE and elicit an allergic response (see Sub-section I 2(A)). Stanworth (1973) has suggested that the property of self-association to form large, structurally complex aggregates may be critical to the allergenicity of proteins at the lower end of the molecular weight range. Although some small allergens, such as melittin in bee venom, do tend to associate others, such as Ra3 and Ra5, do not (Marsh, 1975).

Barrens (1971) noted that many major protein allergens are glycosylated, an observation which prompted him to ascribe the allergenicity of a protein allergen to the proportion of N-glycosidically linked carbohydrates, specifically those linked to ε-amino lysines. This theory was not generally accepted mainly because it failed to account for the allergenicity of unglycosylated protein allergens (for example, the ragweed pollen allergens AgE, Ra4 and Ra5). It is still possible, though, that N-glycosidically linked carbohydrates constitute allergenic determinants in certain glycosylated allergens. Two other features common to many protein allergens have been described; Stanworth (1973) noted that many major allergens are acidic while Roebber et al., (1982) observed that most allergens exist as a number of isoallergenic variants. The significance, if any, of these two features to allergenicity is not known.

Experiments involving the chemical modification of ragweed and honeybee venom allergens (Sub-sections 1 3(B) and 1 3(C)) suggest that conformational features of protein allergens are important in stimulating both an IgE and an IgG response and that the allergenicity of a protein is very sensitive to changes in three-dimensional structure. It is likely, then, that any features which stabilise an allergen will be important to its biological activity. In support of this suggestion Chang and Marsh (1974) showed that ragweed AgE, which is resistant to trypsin, chymotrypsin and papain (King et al., 1967b) is more antigenic and allergenic than the readily degradable rye group 1 allergen. The extreme resistance to denaturation by heat and pH of the allergenic castor bean fraction, CB-1A, was noted in Sub-section I 3(D).

In the light of these studies a number of features common to allergens can be identified including small size, glycosylation, acid pI and a dependence on conformational integrity for their biological activity. None of these features accounts for the unique allergenic properties of this class of proteins (Marsh, 1975; King, 1976).

Other Factors Important in Eliciting an Allergic Response

The allergenicity of a protein does not depend solely on structural features but also on the route and dose of administration and the genetic background of the individual. Low dose immunization is probably a prerequisite of a hypersensitive response since it is now well established that immunization with a minute dose of allergen is one of the conditions required to elicit a persistent IgE response in experimental animals (Ishizaka, 1976). In some cases, such as a bee sting, the amount of allergen administered at each exposure may be relatively large; approximately 7.5 $\mu$g of phospholipase $A_2$, the major bee venom allergen, per sting (Shipolini et al., 1971). Intracutaneous injection of an allergen, though, is an inefficient method of sensitisation since the allergen is not localised in the vicinity of the IgE-producing cells which are associated with the gastro-intestinal and respiratory tracts (Tada and Ishizaka, 1970). Thus, the routes of entry most likely to stimulate an allergic response are inhalation and ingestion (Marsh, 1975). Phills et al. (1972) showed that Ascaris sp., a parasitic worm of the gut, could elicit an IgE response within two weeks of infestation. The short reaction time was probably due to the localisation of the parasite allergens in the vicinity of the intestinal lymph nodes.

An early and comprehensive study by Cooke and Van der Veer (1916) demonstrated that there is a familial predisposition to allergic disease including asthma, allergic rhinitis and atopic eczema. They noted that children are not born with symptoms of allergic disease but inherited the tendency to become allergic. Allergic predisposition was found to be inherited equally from both parents suggesting that it is an

autosomal trait.

In the 1960's McDevitt and colleagues identified immune response genes, linked to the major histocompatibility complex (MHC) of animals, which control the immunoglobulin responses to specific antigens (Benacerraf et al., 1967, Benacerraf and McDevitt, 1972). The effects of these genes, termed immune response (Ir) genes, were only obvious when inbred animal strains were immunised with a structurally simple antigen, synthetic peptides in the case of the earliest studies. Subsequent work showed that the Ir genes are not linked to the major histocompatibility complex but, rather, are part of it (Klein et al., 1981; Klein and Nagy, 1982). The Ir genes map to the I region of the murine MHC, called the H-2 complex, which corresponds to the D region of the human MHC, referred to as the HLA. The products of Ir genes are expressed on the surface of antigen presenting cells and are thought to interact with the processed antigen and ensure correct presentation to T-cells (Grey and Chestnut, 1985).

A number of studies have demonstrated association between specific HLA types and the genetic response to allergens (Marsh et al., 1979; Marsh et al., 1982; Roebber et al., 1985). These studies have largely utilised the Ra5 allergen which, with a molecular mass of only five kDa, is structurally very simple. Administered in low doses this antigen effectively acts like the synthetic peptides used in the original genetic studies since the immune response will be directed to a few, probably only one, immunodominant determinant(s).

Preliminary studies demonstrated an association between the IgE response to Ra5 and the B7 allele of the human MHC (Marsh et al., 1973). This association was shown to be secondary to a primary association between the IgG and IgE responses against Ra5 and the HLA-Dw2 allele (Marsh et al., 1982). The HLA-Dw2 allele is now regarded as a good marker for immune responsiveness to Ra5. The apparent association with

HLA-B7 is probably the result of linkage disequilibrium between the B7 and Dw2 alleles. Other examples of HLA associations with the immune response to specific allergens include: ragweed allergen six (Ra6) with HLA-DR5 (Marsh et al., 1986) and a rye-grass, Lolium perennae, allergen (Lol pIII) and HLA-Dw3 (Ansari et al., 1986). In addition to regulation by Ir genes the IgE response is subject to genetic control not linked to the MHC and which does not act in an antigen-specific manner (Levine and Vaz, 1970).

It has been concluded that there is no common, inherent feature of protein allergens that accounts for their biological properties. A protein is allergenic because of the favourable combination of the factors discussed (Marsh, 1975; King, 1976).

Future Approaches to the Investigation of Protein Allergenicity

It is important to emphasise that historically the analysis of allergenic proteins has only taken into account gross physicochemical properties such as charge, size and sequence. Due to technical limitations it was not possible to accurately determine the structural features recognised by B and T cells and thereby begin to understand the cellular mechanisms involved in the control of the IgE response. A more subtle molecular analysis of the relationship between structure and function in eliciting an immune response may reveal structural features common to allergens which previous analyses were too crude to detect.

In the past decade considerable work has gone into the elucidation of protein antigenic determinants recognised by B or T-cells (Atassi et al., 1984; Benjamin et al., 1984). These advances have been made possible by technical breakthroughs such as the development of monoclonal antibodies and the ready availability of synthetic peptides. Although this work has involved non-allergenic proteins the results are considered to be applicable to allergens since, at the current level of knowledge, the latter may simply be regarded as a subset of protein antigens distinguished by their biological activity. Despite the large amount of data which has been generated few firm conclusions have been reached principally because all studies of protein antigenicity are operationally biased, that is, individual techniques used to identify antigenic sites favour particular results and interpretations of the data (for a discussion of this problem see Van Regenmortel, 1986).

Currently there are two opposing views of what constitutes an antigenic determinant; one view is that a protein has only a few, discrete sites which are inherently capable of stimulating an immune

response irrespective of the immunized host (for a review of this position see Atassi, 1984). The opposing view is that most of the accessible surface of a globular protein is potentially antigenic, the response to individual sites being determined by the genetic background of the host. According to this view an antigenic determinant can only be defined with respect to a particular serum (for a review of this position see Benjamin et al., 1984).

Despite the considerable contention in this area at least two conclusions are generally held to be true; B-cells tend to recognise discontinuous determinants, assembled topographic determinants composed of residues brought together by the folding of the protein, whilst T-cells recognise a largely separate group of mainly continuous determinants composed of sequentially linked amino acid residues. B-cell determinants are, therefore, much more dependant on the integrity of the three-dimensional conformation than are T-cell determinants, an observation supported by the reduced allergenicity of chemically modified allergens (see Sub-sections I 3(B) and I 3(C)). From a practical viewpoint this observation is important since it is easier to identify continuous determinants and to understand the effects of specifically engineered amino acid substitutions on their biological activity when no account need be taken of conformational changes. The relevance of this observation to allergen research stems from the fact that an important use for purified, characterised allergens may be the development of specifically modified derivatives capable of inducing a T-cell population which can suppress the IgE response against the native allergen. This approach to immunotherapy will be discussed in the Concluding Chapter, Section C3.

# SECTION 4

# CASTOR BEAN STORAGE PROTEINS

# SECTION 4

# CASTOR BEAN STORAGE PROTEINS

## Storage Proteins of the Castor Bean

During the development of the castor bean protein is deposited rapidly in the endosperm between 10 and 35 days after pollination (Gifford et al., 1982). This protein is stored in intracellular, membrane-bound organelles in the endosperm (Tully and Beevers, 1976; Youle and Huang, 1976), a feature shared with many other plant species (Lott, 1980). At the time of germination this protein is hydrolysed (Gifford et al., 1983). The protein bodies contain three classes of storage protein; 7S lectins (Olsnes et al., 1974; Tully and Beevers, 1976; Youle and Huang, 1976), the 2S albumins (Tully and Beevers, 1976; Youle and Huang, 1976; Li et al., 1977; Sharief and Li, 1982) and the 11S crystalloids (Tully and Beevers, 1976; Youle and Huang, 1976; Gifford and Bewley, 1983; Gifford and Bewley, 1984). Since these are the major groups of proteins in mature castor beans and since previous work suggested that at least one class of castor bean storage proteins, the 2S albumins, are allergenic (Sub-section I 3(D)) the storage proteins will be described in detail, beginning with the crystalloids.

## The 11S Crystalloids

Osborne (1895) classified plant proteins into groups on the basis of their extraction in a series of solvents: water (albumins), dilute salt solutions (globulins), aqueous 70% ethanol (prolamins) and dilute alkali (glutelins). Within the 11S globulins two categories can be distinguished: those that are completely soluble in SDS or urea, for example the legumins, and those which also require salt to fully dissolve, the crystalloids (Gifford and Bewley, 1983).

The crystalloids constitute 70-80% of the endosperm storage proteins in castor beans (Tully and Beevers, 1976; Gifford et al., 1982). Youle and Huang (1976) analysed the crystalloids by tube gel electrophoresis and found several proteins in the molecular mass range 50-60 kDa. In a similar study Tully and Beevers (1976) also found several proteins in the 50-60 kDa range but with other components, some as small as 17 kDa. On reduction, the major 65 kDa component yielded polypeptides of 32 kDa and 15.8 kDa. Tully and Beevers proposed that the unreduced crystalloids were composed of two small subunits and one large subunit. Under non-denaturing conditions the crystalloid holoprotein had previously been shown to have a molecular mass of about 330 kDa (Joubert, 1955; Derbyshire et al., 1976) and is, therefore, probably a hexamer of the 65 kDa subunit (itself composed of two polypeptides).

A more exact study undertaken by Gifford and Bewley (1983) using R. communis cv Hale revealed at least six oligomers in the molecular mass range 49-53 kDa. The individual oligomers were present in unequal amounts. Each oligomer existed as a number of isoelectric variants, even within a single seed implying that the effect was not simply due to

variation within the seed population. Reduction of the oligomers yielded two groups of polypeptides in the molecular mass ranges, 29-34 kDa and 20.5 to 23.5 kDa. In general the large subunits had acidic pIs and the small subunits had basic pIs although there was some overlap between the two groups. Gifford and Bewley related acidic and basic polypeptides to the oligomers from which they came. On this basis they proposed a structural model in which one basic subunit associated with one acidic subunit to form an oligomer, in contrast to the model of two basic polypeptides and one acidic polypeptide proposed by Tully and Beevers (1976). The latter estimated molecular weight using tube gels which were probably not as accurate as SDS-PAGE used by Gifford and Bewley. The current model for the structure of the crystalloid protein, then, is a holoprotein formed by the non-covalent association of six subunits. The subunits, in turn, are composed of one acidic and one basic polypeptide joined by disulphide bonds. This model is summarised in Figure I 2.

The synthesis of the crystalloid complex has been studied by Bewley and coworkers. Gifford et al. (1982) using light microscopy and protein staining procedures showed that the crystalloids were first synthesised 25 days after pollination whilst the matrix proteins were not visible for another five days. All the components of the crystalloid complex were synthesised at the same time while the matrix proteins showed variation in the time of deposition. Unlike the crystalloids, individual components of the hordeins, globulin storage proteins of barley, have been shown to accumulate differentially during seed development (Rahman et al., 1982). Subsequently Gifford and Bewley (1984) showed that the crystalloids are synthesised as high molecular mass precursors of 50-60 kDa which contain an acidic subunit and a basic subunit. Examples of globulin precursors with a similar composition are known from other plant species; oat (Brinegar and Peterson, 1982), pea

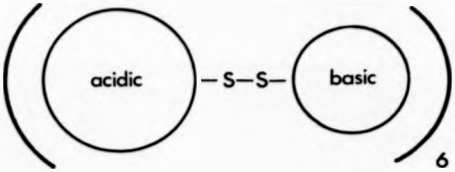
Figure I 2 Structural models for the three classes of castor bean storage proteins

The structure of each protein class under denaturing conditions is shown inside the brackets. The numbers outside the brackets indicate the number of monomers which associate under non-denaturing conditions where this value has been determined. In the case of the 2S albumins this value has not been determined, an unspecified value, n, being assigned to this class of proteins.

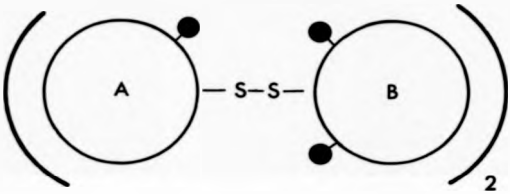
A description of the structure of each class of storage protein is given in the appropriate Sub-section of the Introduction: 11 S crystalloids, Sub-section I 3(B); 7S lectins, Sub-section I 3(C); 2S albumins, Sub-section I 3(D).

The shaded circles represent carbohydrate groups.

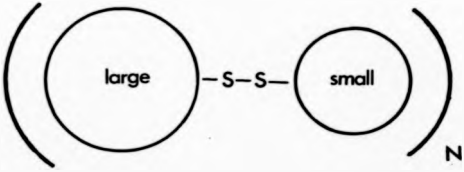
11 S CRYSTALLOIDS




7S LECTINS




2S ALBUMINS



(Chrispeels et al., 1982) and soybean (Sengupta et al., 1981). The variation in crystalloid subunits was attributed to a multigene family and not post-translational processing since unprocessed precursors also existed as a range of isoelectric variants. Multigene families are a common feature of seed storage proteins: for example, zeins in Zea mays (Langridge and Feix, 1983) and glutenins in wheat, Triticum aestivum (Thompson et al., 1983).

That the crystalloids are mobilised at the onset of germination and, hence, are probably storage proteins was demonstrated by Gifford et al., (1983). Two days after the onset of germination the protein bodies coalesced to form a central vacuole, a process which took two days. Concurrently the crystalloids and 2S albumins were hydrolsed although the lectins mobilised very slowly.

Protein bodies are also found in the cotyledons and, interestingly, here only 10% of the storage protein is crystalloid (Kermode et al., 1985). Both the lectins are absent from the cotyledons whilst other proteins, not seen in the endosperm, are present. Mobilisation of cotyledon storage proteins is completed after 48 h, at which time the mobilisation of endosperm storage proteins is only beginning (Gifford et al., 1983). It is likely that the cotyledons act as a nutrient store for the initial stages of germination.

## Castor Bean Lectins

The lectins are undoubtedly the best characterised of the castor bean storage proteins (for a full account see the reviews of Balint, 1974; Olsnes and Pihl, 1982). The main interest in this group of proteins is in using the toxic lectin, ricin, as an immunotoxin. Despite the wealth of data which has been accumulated describing the structure and properties of the lectins only a brief review will be given here as there is no evidence either from previous studies or from this study to suggest that the lectins are allergenic.

The castor bean lectins are composed of an agglutinin and the toxin, ricin, both of which exist as a number of variants depending on the variety of the bean from which they are isolated (Balint, 1974). The toxin and the agglutinin are generally separated by elution from a Sepharose column with N-acetylgalactosamine and galactose, respectively (Butterworth and Lord, 1983). The molecular weights of the two lectins, as determined by sedimentation velocity, gel filtration and electrophoresis fall within the ranges, 54,000-65,000 for the ricins and 110,000-140,000 for the agglutinins (Gurtler and Horstmann, 1973; Olsnes *et al*., 1974; Gonatas *et al*., 1977; Wei and Kah, 1978; Butterworth and Lord, 1983).

Ricin is a heterodimer composed of an A-chain and a B-chain joined by a disulphide bond. Boiling the agglutinin in SDS generates a molecule of about the same molecular weight as ricin (Cawley and Houston, 1979) which is composed of an A' and a B' chain. The agglutinin is therefore thought to be a tetramer of two ricin-like dimers. The two dimers are held together by non-covalent interactions (Suralia *et al*., 1976). The model currently accepted for the structure of ricin and the agglutinin is shown in Figure I 2.

The amino acid sequences for both chains of one form of ricin, ricin D, were determined by Funatsu and colleagues (Kimura et al., 1977; Fanatsu et al., 1978a & b; Yoshitake et al., 1978; Funatsu et al., 1979; Kimura and Funatsu, 1981). There is only a partial amino acid sequence for the agglutinin (Cawley et al., 1978). N-terminal sequence comparison of the first 9 amino acid residues of the ricin and agglutinin A-chains revealed that they were identicial as were the first 19 amino acid residues of the B-chains (Cawley et al., 1978). Both chains of both proteins are glycosylated (Gonatas et al., 1977; Gurtler and Horstmann, 1973).

The lectins first appear in the developing seed about 20 days after pollination (Gifford et al., 1982). Immunoprecipitations studies using antibodies raised against purified agglutinin A and B chains identified what was apparently a single lectin precursor of molecular weight 59,000 (Butterworth and Lord, 1983). Subsequent studies involving the isolation and sequencing of cDNA encoding the precursors to the lectins have demonstrated that ricin and the agglutinin are translated as separate precursors composed of one A-chain and one B-chain joined by a linker peptide and preceded by a signal sequence (Lamb et al., 1985; Roberts et al., 1985). Sequence comparison between the two clones has revealed the A-chains to be 93% homologous with each other and the B-chains to 84% homologous with each other.

Ricin is a member of a large group of ribosome-inhibiting proteins (Barbieri and Stirpe, 1982). The A-chain is the active component which irreversibly inactivates the ribosome while the B-chain, by means of its sugar-binding group, binds to exposed sugar residues, probably galactose, and thereby mediates endocytosis. Consequently, a possible role for ricin and related proteins in plants is as a defence mechanism against grazing (Janzen, 1983). Alternatively the lectins may simply be storage proteins (Youle and Huang, 1978a). It is also possible that they serve a combination of both functions.

## The 2S Albumins

In their early studies on the composition of castor bean storage proteins Tully and Beevers (1976) identified two major, unglycosylated albumins of molecular masses 10.3 and 12.5 kDa. A concurrent study by Youle and Huang (1976) identified at least one 14 kDa albumin protein. As the protein was not degraded during the first five days of germination they inferred that it did not have a storage role. In a subsequent study, specifically of the 2S albumins, Youle and Huang (1978) isolated a 2S fraction composed of several proteins of molecular weight masses 12 kDa. Contrary to their earlier observation Youle and Huang found that these proteins were rapidly degraded at the onset of germination which, together with a high content of glutamine/glutamate (40%), led them to conclude that the 2S albumins are really storage proteins. This conclusion was supported by the earlier work of Stewart and Beevers (1967) who found that 40% of the amino acids transported from the endosperm to the growing shoot were glutamine, consistent with the amino acid composition of the 2S albumins.

In a study on the deposition of matrix and crystalloid proteins Gifford et al. (1982) identified three or four unglycosylated 2S albumins of molecular mass about 14 kDa. The synthesis of these proteins began concurrently with that of the lectins at about 20 to 25 days after pollination. In a complementary study of storage protein breakdown in castor bean endosperm Gifford et al. (1983) found that both the 2S albumins and the crystalloids were mobilised concurrently at between two and four days after the onset of germination, consistent with their role as storage proteins. The loss of protein was accompanied by an increase in the activity of three proteases; one

carboxypeptidase and two SH-dependant aminopeptidases.

Li and colleagues isolated and sequenced a 2S albumin storage protein from castor beans (Li et al., 1977; Sharief and Li, 1982). The protein was a heterodimer composed of a large subunit, 7 kDa, and a small subunit, 4 kDa. The sequence was similar in amino acid composition to the 2S fraction characterised by Youle and Huang (1978a), having a high percentage of glutamic acid and cysteine. The amino terminal of the large subunit was homologous with part of the Bowman-Birk serine protease inhibitor of lima bean (Tan and Stevens, 1971) including the active site of trypsin inhibition. The trypsin inhibitory activity of the 2S albumin was not tested. The structure of the 2S albumin isolated by Li is shown in Figure I 2.

The subunit composition of the 2S albumin isolated by Li and colleagues is unusual in that the earlier work of Tully and Beevers (1976) suggested that neither of the two major 2S albumins isolated by them could be reduced to yield subunits. In addition, since evidence will be presented in Section 5 of the Results and Discussion Section to show that the protein isolated by Li is, as the sequence suggests, a trypsin inhibitor, it is highly unusual to find a plant serine protease inhibitor composed of covalently linked subunits (see Section I 5 for a discussion of plant serine protease inhibitors). A literature search by the author failed to find a precedent for this structural organisation in the relevant classes of inhibitor.

Small, cysteine-rich, albumin storage proteins, distinct from the various classes of protease inhibitors have been identified in a number of other plant species (reviewed by Youle and Huang, 1981) including: soybean (Hill and Breidenbach, 1974), pea (Gatehouse et al., 1985) and oilseed rape (Lonnerdal and Janson, 1972). It has been proposed that these proteins are a distinct class of storage proteins which act as sulphur reserves for the germinating plant (Youle and Huang, 1981). In

common with the castor bean 2S albumin sequenced by Sharief and Li (1982), the 2S proteins of pea and oilseed rape are both covalently linked dimers.

# SECTION 5

# SERINE PROTEASE INHIBITORS FOUND IN PLANTS

# SECTION 5

# SERINE PROTEASE INHIBITORS FOUND IN PLANTS

## A Castor Bean Serine Protease Inhibitor may be an Allergen

Evidence will be presented in Section 3 of the Results and Discussion chapter that the 2S albumin purified and sequenced by Li and colleagues (Li et al., 1977; Sharief and Li, 1982) and which is also a serine protease inhibitor (Section 5 of the Results and Discussion chapter) is a putative castor bean allergen. In consequence, a brief review will be given of plant serine protease inhibitors.

## General Properties of Serine Protease Inhibitors Found in Plants

Most plant protease inhibitors which have been identified are specific for serine proteases. Only a few have been identified which are specific for thiol proteases (Reddy et al., 1975), metalloproteases (Fritz et al., 1974) or acid proteases (Goldstein et al., 1973).

In his review Ryan (1981) noted that plant serine protease inhibitors are typically of low molecular weight, in the range 8,000-20,000, contain a high percentage of cysteine residues, often one cysteine per 8-10 amino acid residues, and tend to be deficient in methionine, tryptophan and histidine but rich in glutamic and aspartic acids, serine and lysine residues. Plant protease inhibitors are not glycosylated. Non-covalent association to form specific size classes of multimers is a common feature; for example, inhibitor 2 of potatoes is a 21 kDa homodimer assembled from a group of four different isoinhibitor monomers (Bryant et al., 1976). The existence of isoinhibitor variants, differing in size, amino acid composition and pI, is also a common feature (Melville and Ryan, 1972; Bryant et al., 1976; Gennis and Cantor, 1976). Serine protease inhibitors are typically found in the storage organs of plants where they may constitute a large percentage of the protein; for example potato protease inhibitors 1 and 2 may comprise up to 7% of the soluble protein in potato tubers depending on the variety (Ryan et al., 1976).

Despite claims to the contrary the exact mechanism of inhibition has not been determined (Hunkapiller et al., 1979; Fujinaga et al., 1982). X-ray crystallographic studies of enzyme-inhibitor complexes have shown that the reactive site of the inhibitor acts as a transition state analogue giving an excellent fit stabilised by non-covalent forces

such as salt bridges and Van der Waals interactions (Fersht, 1985). In general, the $P_1$ residue of the reactive site corresponds to the amino acid specificity of the protease inhibited; when $P_1$ is lysine or arginine trypsin is inhibited, with tyrosine, phenylalanine, tryptophan, leucine or methionine at the $P_1$ site chymotrypsin is inhibited (Laskowski and Kato, 1980). The stoichiometry of the enzyme-inhibitor complex is 1:1.

Serine protease inhibitors can be multiheaded in that they have more than one inhibitory site (Rhodes et al., 1960). Multiheadedness may arise by the non-covalent association of inhibitory monomers as in the case of potato inhibitor 1, a 40 kDa tetramer of isoinhibitor monomers (Melville and Ryan, 1972). Another way of achieving multiheadedness is by gene duplication to give a single polypeptide organised into two homologous domains each with an inhibitory site. This arrangement is common to all members of the legume Bowman-Birk family of serine protease inhibitors.

## The Classification of Serine Protease Inhibitors Found in Plants

The classification of serine protease inhibitors has largely been based on sequence homology and topological relationships involving the disulphide bonds (Laskowski and Kato, 1980). The best example of an inhibitor family defined by these criteria is the Bowman-Birk family mentioned above whose members are found in the seeds of leguminous plants. The monomers are typically small, less than 12 kDa, and rich in cysteine, the inhibitor from soybean has 14 cysteines out of a total of 71 amino acid residues (Odani and Ikenaka, 1973). Isolation and sequencing of the lima bean inhibitor revealed that the amino acid and carboxyl terminal halves were homologous (Tan and Stevens, 1971). Subsequently a number of inhibitors of this class were isolated and sequenced including four examples from soybean; BBI (Odani and Ikenaka, 1972), D2 and E1 (Odani and Ikenaka, 1976 and 1978) and C2 (Odani and Ikenaka, 1977) and one from garden beans (Wilson and Laskowski, 1975).

This sequence data allowed Odani and Ikenaka (1978) to devise a possible evolutionary pathway for the Bowman-Birk serine protease inhibitors. An ancestral trypsin inhibitor gene was envisaged as having duplicated, producing a double-headed inhibitor specific for trypsin, like the D2 inhibitor of soybean (Odani and Ikenaka, 1978a), which subsequently underwent mutation to produce an inhibitor with two different specificities, for example the garden bean inhibitor which is specific for both trypsin and elastase (Wilson and Laskowski, 1975).

Odani and Ikenaka (1973) had also previously determined the disulphide bond pattern of BBI; the amino and carboxyl terminii showed almost exactly homologous disulphide bond patterns reflecting the sequence homology between the two. Each half of the molecule is

arranged into three disulphide-bonded loops with the reactive site located on the largest, outermost loops at diametrically opposed positions (Figure I 3). The separation of the two inhibitory sites may help to prevent steric hindrance since the inhibitor can complex with both trypsin and chymotrypsin simultaneously (Birk, 1968).

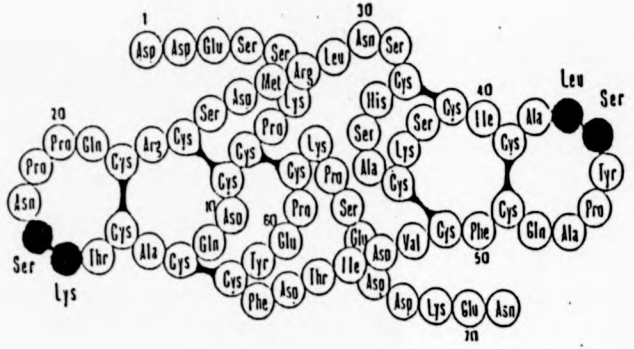
The first plant protease inhibitor to be characterised was another soybean inhibitor originally isolated by Kunitz (1947a and 1947b) which still bears his name and which forms the basis of another serine protease inhibitor family. The Kunitz inhibitor is composed of 181 residues with a molecular weight of about 20,000 and is, therefore, twice as large as a typical Bowman-Birk inhibitor. Kunitz inhibitors have few disulphide bonds, two in the case of the original soybean inhibitor. At the time of writing their review Laskowski and Kato (1980) stated that no other example of a Kunitz inhibitor was known in legumes although homologues had been found in winged bean (Kortt, 1979) and barley (Yoshikawa et al., 1976; Mikola and Suolina, 1971). Subsequently a Kunitz inhibitor has been isolated from the seeds of Erythrina latissima, a leguminous shrub found in tropical regions (Joubert et al., 1985).

The remaining two inhibitor families are based on two groups of inhibitors found in potatoes. The members of the potato 1 family are 40 kDa tetramers formed by the non-covalent association of a group of monomeric isoinhibitors (Melville and Ryan, 1972). The potato 2 inhibitor family is more complex. A 10 kDa protein inhibitor of trypsin and chymotrypsin was partially sequenced by Iwasaki et al. (1972). Bryant et al. (1976) subsequently isolated four such monomeric isoinhibitors each of molecular weight about 10,500 which associated to form homodimers. Two low molecular weight (about 6,000) serine protease inhibitors, an inhibitor of trypsin, PTI-1, and an inhibitor of chymotrypsin, PCI-1, were isolated from potato, sequenced, and were

Figure I 3 The disulphide bond pattern of a Bowman-Birk serine protease inhibitor

Odani and Ikenaka (1973) determined the disulphide bond assignments of a Bowman-Birk serine protease inhibitor isolated from soybean. The disulphide bond pattern is shown in Figure I 3. The sites of inhibition of trypsin (lys-ser) and chymotrypsin (leu-ser) are indicated by shaded circles.

The amino acid residues are numbered from the amino terminus.


1
10
20
30
40
50
60
70
Asp Asp Glu Ser Ser Leu Asn Ser
Met Arg Lys
Asn Ser
Cys
His
Cys
Leu
Ser
Ile Ala
Ser
Ser
Lys
Cys
Pro
Gln
Cys
Arg
Cys
Pro
Cys
Lys
Ala
Cys
Tyr
Pro
Asn
Cys
Pro
Ser
Cys
Gln
Ala
Asn
Gln
Glu
Glu
Val
Cys
Phe
Ser
Thr
Ala
Cys
Tyr
Asn
Ile
Thr
Asp
Lys
Glu
Asn
Lys
Cys
Phe

found to be 83% homologous to each other (Hass et al., 1982). The inhibitors were also homologous with the partial sequence of inhibitor 2 (Iwasaki et al., 1972). Of particular interest was the homology between one of the low molecular weight inhibitors, PCI-1, and the sequence of the amino terminal 18 amino acids of a metallocarboxypeptidase inhibitor determined by Hass et al. (1976).

These observations suggested two possibilities; first, that inhibitors directed toward two distinct classes of protease, the serine endopeptidases and the metallocarboxypeptidases, may have evolved from a common ancestor and, second, that the small serine protease inhibitors of the potato 2 family are derived by post-translational processing from the larger members. In support of the latter possibility is the work of Sanchez-Serrano et al. (1986); the nucleotide sequence of inhibitor 2 shows a region of complete homology, at the amino acid level, with PCI-1 and a region of 84% homology with PTI-1. Further, Northern blot analysis of potato mRNA using an inhibitor 2 cDNA clone as probe revealed only one transcript suggesting that PCI-1, PTI-1 and inhibitor 2 are all transcribed from the same gene.

The Physiological Role of Protease Inhibitors Found in Plants.

There is no clear evidence to indicate the normal physiological role of plant serine protease inhibitors or of plant protease inhibitors in general. A commonly held view is that they inhibit endogenous proteases and prevent premature degradation of seed storage proteins (Ryan, 1981). Hobday et al. (1973) and Pustzai et al. (1972) have noted that germination is accompanied by a gradual decline in the level of trypsin inhibitors in the cotyledons of pea, Pisum sativum. and beans, Phaseolus vulgaris. The first problem in assessing this idea is that often it is not known if an inhibitor is capable of inhibiting an endogenous protease since either the possibility has not been tested or there is no known protease from the appropriate species. A number of species have now been shown to have proteases inhibited by the endogenous inhibitor: for example, corn (Reed and Penner, 1978), wheat (Preston and Kruger, 1976) and rice (Horiguchi and Kitagishi, 1971).

Attempts have been made to determine if removal of an endogenous inhibitor results in increased proteolytic activity. Royer (1975) showed that removal of a trypsin inhibitor from cowpea extracts by means of an immobilised inhibitor column resulted in a 2.5 fold increase in casein hydroysis, although the enzyme responsible was not characterised. Shain and Mayer (1968) showed that endogenous trypsin inhibitory activity in lettuce seeds completely disappeared on the first day of germination with a subsequent 50-fold increase of trypsin-like activity over the next two days, implying a causal relationship between the two events. While this relationship may hold in some instances it is probably not a generally applicable explanation of the normal role of plant protease inhibitors in view of the data discussed below.

Chrispeels and Boulter (1975) showed that the rapid degradation of storage protein accompanying germination in mung beans was dependant on a sulphydryl protease. The enzyme was synthesised de novo and localised in the protein bodies. Enzyme synthesis increased 25-fold in the first five days of germination (Chrispeels et al., 1976). The sulphydryl protease, vicilin peptidohydrolase, was subsequently purified to homogeneity and was shown to be a 23 kDa protein of pI 3.75 which readily hydrolysed the storage protein, vicilin (Baumgartner and Chrispeels, 1977). Endogenous inhibitors of vicilin peptidohydrolase and of trypsin were known to exist in mung beans; two sulphydryl protease inhibitors of molecular weights 12,000 and 2,000 and a serine protease inhibitor of molecular weight 12,000 (Baumgartner and Chrispeels, 1976). In the same paper it was demonstrated that inhibitory activity slowly disappeared from the first day onwards but endopeptidase activity remained low until the third day of germination when it rose sharply. Thus, the kinetics of the two phenomena did not appear to be causally related. Further, fractionation of the cellular organelles showed that the inhibitory activities directed against the sulphydryl proteases were associated with the cytosol rather than with the protein bodies while trypsin-inhibitory activity was associated with both the cytosol and the protein bodies. In the light of these results Baumgartner and Chrispeels (1976) suggested that the endogenous inhibitor served to protect the cytoplasm from proteases released from ruptured protein bodies.

Gennis and Cantor (1976) proposed a novel function for protease inhibitors isolated from black-eyed peas. They isolated two double-headed, serine protease inhibitors both with molecular weights of 8,000, one specific for trypsin and one for trypsin and chymotrypsin. The latter could be isolated as a complex with an endogenous trypsin-like enzyme which they also purified from mung beans. It was found that the

enzyme was less stable when stored in solution in the absence of the inhibitor. They proposed that, *in vivo*, the inhibitor may serve to stabilise the protease until required in germination, at which time the inhibitor would be specifically degraded or the complex dissociated.

Another possible role for plant protease inhibitors is in the defense response against invading pathogens. Numerous studies by Ryan and colleagues in a range of plant species have established that mechanical damage to a leaf induces the synthesis of the protease inhibitors usually only found in storage organs (Ryan, 1974; McFarland and Ryan, 1974; Bishop *et al*., 1981). The synthesis of protease inhibitors is induced by a systemic signal, carbohydrate components of the damaged cell walls (Ryan *et al*., 1981). In tomatoes the newly synthesised inhibitors are known to be deposited in the central vacuole of the cell (Walker-Simmons and Ryan, 1977) and it has subsequently been shown that tissue damage induces a signal, distinct from that which induces synthesis of the protease inhibitors, which alters the properties of isolated leaf protoplasts, making them much more sensitive to osmotic shock. The nature of the second signal is not known but it may play a role in releasing inhibitors sequestered in the central vacuole. Once induced, protease inhibitors are thought to inhibit the digestive enzymes of predatory insects and thereby discourage grazing (Green and Ryan, 1972; Janzen *et al*., 1977).

## Future Research

The most recent work in this area has been the isolation and sequencing of cDNA clones for a variety of plant serine protease inhibitors: BBI from soybean (Hammond *et al.*, 1984), inhibitors 1 and 2 from tomato (Graham *et al.*, 1985a and 1985b) and inhibitor 2 from potato (Sanchez-Serrano, 1986). Isolation of the corresponding genomic clones will help identify the regions controlling the expression of these proteins, some of which exhibit both tissue-specific and environmental control.

# CHAPTER 2

# MATERIALS AND METHODS

M1 Materials

All materials used were of the highest analytical grade available. The sources of specific reagents are given below.

Amersham International plc, Amersham, Buckinghamshire: all radiochemicals, $^{35}$S-labelled protein molecular weight kit, rabbit reticulocyte lysate translation kit, biotin-streptavidin kit for Western blots, chromatofocusing polybuffer and matrix.

Anderman and Co. Ltd., 145, London Road, Kingston-upon-Thames, Surrey: Schleicher and Schull nitrocellulose filters.

BDH Chemicals Ltd., Broom Road, Poole, Dorset: acrylamide, ammonium persulphate, Schiff's reagent, sucrose, sodium hydroxide, citric acid, sodium azide, 2-mercaptoethanol, phenol crystals, sodium acetate, potassium chloride, zinc chloride, sodium pyrophosphate, sodium hydroxide, bromophenol blue, ammonium acetate, N,N,N,N'-tetramethylene diamine (TEMED).

Beckman-RIIC Ltd., High Wycombe, England: non-aqueous scintillant.

BRL, PO Box, 145, Science Park, Cambridge, UK: oligo(dT)-cellulose, RNAse H.

Calbiochem, Behring Diagnostics, La Jolla, California, USA: Aquacide.

Cecil Instruments Ltd., Milton Industrial Estate, Cambridge, England: Model CE 292 digital ultra-violet spectrophotometer.

Clause (UK) Ltd., New Bath Road, Charvil, England: castor bean (var. Impala) seeds.

Eastman Kodak, Rochester, New York, USA: N,N'-methylene bisacrylamide, X-ray film (X-Omat S), photographic film (Panatomic-X).

Fisons Scientific Apparatus, Loughborough, Leicester: dimethyl sulphoxide, ammonium hydroxide, formaldehyde, glycine, hydrogen peroxide, urea, glycerol, sodium chloride, diaminoethanetetra-acetic acid (EDTA), sodium dodecyl sulphate (SDS), magnesium chloride, calcium chloride.

GIBCO, Grand Island, New York, USA: complete and incomplete freund's adjuvant.

Ilford Ltd., Mobberley, Cheshire: FF Contrast photographic developer.

Kodak Ltd., Hemel Hempstead, England: photographic fixers and developers.

Life Sciences Inc., 2900 72$^{nd}$ Street, North Saint Petersburg, Florida, USA: avian myeloma virus reverse transcriptase.

May and Baker Ltd., Dagenham, England: concentrated hydrochloric acid, glacial acetic acid, trichloroacetic acid.

New England Biolabs, 32, Tozer Road, Beverley, Massachusetts, USA: DNA polymerase 1.

Olac, Shaw's Farm, Bicester, England: white, lop-eared rabbits.

P and S Biochemicals Ltd., 38, Queensland Street, Liverpool: RNAse inhibitor (RNAsein).

Paterson Products Ltd., Rainham Road South, Dagenham, England: Acutol photographic developer.

Pharmacia (GB) Ltd., London: Sephadex G-50, DEAE-Sephacel, low molecular weight calibration kit for SDS-PAGE, protein A sepharose, deoxyribonucleoside triphosphates (under the name of the subsidiary, P and L Biochemicals Ltd.).

Plant Breeding Institute, Maris Lane, Trumpington, England: S1 nuclease.

Sigma Chemcial Co., Ltd., Fancy Road, Poole, Dorset: dithiothreitol, iodoacetamide, Coomassie brilliant blue R, silver nitrate, periodic acid, sodium metabisulphite, protein A, bovine serum albumin, Triton X-100, 3'-diaminobenzidine, N-chlorosuccinimide, V8 protease, Tris base, Nonidet P-40, phenylmethylsulphonyl fluoride (PMSF), p-Chloromercuribenzoic acid (PCMB), 1,10-phenanthroline, 2,5-diphenyloxazole, agarose, imidazole, N-benzoyl-L-arginine ethyl ester (BAEE), N-benzoyl-L-tyrosine ethyl ester (BTEE), trypsin (T-8003), $\alpha$-chymotrypsin (C-4129), lima bean trypsin inhibitor, molecular weight calibration kit for non-denaturing gels.

Whatman Labsales Ltd., Maidstone, Kent: filter paper (3 MM and GF/C), CM-cellulose.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The discontinuous buffer system of Laemmli (1970) was employed, using slab gels of 22 x 20 x 0.15 cm. The stock solutions used in preparing the resolving gel mixture were: acrylamide: bisacrylamide, 30 : 0.8% (w/v); 3 M Tris-HCL (pH 9.0); SDS, 10% (w/v).

The stock solutions were mixed in the following proportions:

| | ml |
|---|---|
| Acrylamide : bisacrylamide | x |
| Tris-HCL (pH 9.0) | 16 |
| SDS | 0.8 |
| Distilled water | to final volume |
| Final Volume | 80 |

where x is the volume of stock acrylamide required to give the desired final concentration (40 ml for a 15% gel and 28 ml for a 10% gel).

Polymerization of the resolving gel mixture was by addition of 200 µl 10% (w/v) ammonium persulphate and 32 µl TEMED. The gel mix was degassed before pouring. Immediately after pouring the resolving gel was overlaid with water-saturated isobutanol and left for at least two hours to polymerise.

The top of the resolving gel was washed with distilled water prior to the addition of the stacking gel. The stock solutions used in

preparing the stacking gel mixture were: acrylamide : bisacrylamide, 30 : 0.8% (w/v); 0.5 M Tris-HCL (pH 6.8); SDS, 10% (w/v). The following volumes of the stock solutions were mixed:

| | ml |
|---|---|
| Acrylamide : bisacrylamide | 4.0 |
| 0.5 M Tris-HCL (pH 6.8) | 3.2 |
| SDS | 0.25 |
| Distilled water | 17.5 |
| Total Volume | 25 |

In every case the acrylamide concentration of the stacking gel was 5% (w/v). Polymerisation of the stacking gel mixture was by addition of 150 µl of 10% (w/v) ammonium persulphate and 12.5 µl TEMED. The gel mixture was degassed before pouring. After pouring a plastic gel comb was inserted and the stacking gel allowed to set for at least one hour. Once the gel had set the comb was removed and the gel mounted in the electrophoresis tank. The composition of the electrophoresis running buffer was: 25 mM Tris-HCL, pH 8.8, 192 mM glycine and 0.1% (w/v) SDS.

Samples were loaded in the following buffer:

| | |
|---|---|
| Tris-HCL (pH 8.8) | 35 mM |
| Sucrose | 90 mM |
| EDTA | 1 mM |
| Bromophenol blue | 0.01% (w/v) |

Once in solution samples were boiled for two min. When required reduction and acetylation of the samples was by addition of one µl of 0.5 M dithiothreitol and one µl of 0.5 M iodoacetamide, respectively. Electrophoresis was carried out at room temperature at 18-22 mA constant current for 12-16 h until the bromophenol blue dye front had reached the bottom of the gel. The gel was then removed for staining (see Section M4).

SDS-PAGE gels were routinely run with protein markers purchased as a kit from Pharmacia Ltd. The molecular weight standards were:

| | molecular mass kDa |
|---|---|
| Phosphorylase b | 94 |
| BSA | 67 |
| Ovalbumin | 43 |
| Carbonic Anhydrase | 30 |
| Soybean Trypsin Inhibitor | 20.1 |
| α-Lactalbumin | 14.4 |

The following $^{14}C$-labelled molecular weight markers were used in the case of gels which were to be exposed to film:

| | molecular mass kDa |
|---|---|
| Myosin | 200 |
| Phosphorylase b | 92.5 |
| Bovine serum albumin | 69 |
| Ovalbumin | 46 |
| Carbonic anhydrase | 30 |
| Lysozyme | 14.3 |

## Non-Denaturing Gel Electrophoresis

Linear gradient gels of 4-30% (w/v acrylamide) were poured as slab gels of 17 x 17 x 0.15 cm. The 4% gel mixture was composed of:

| | ml |
|---|---|
| Acrylamide : bisacrylamide | 4.6 |
| 3 M Tris-HCL, pH 8.8 | 3.75 |
| Distilled water | 20.4 |

where the acrylamide : bisacrylamide stock was 25% : 1.25% (w/v). Polymerisation of the 4% gel mixture was by addition of 150 µl 10% (w/v) ammonium persulphate and 17.5 µl TEMED.

The 30% gel mixture was composed of:

| | |
|---|---|
| Acrylamide : bisacrylamide | 14.4 |
| 3 M Tris-HCL, pH 8.8 | 3.75 |
| 30% (v/v) Glycerol | 10.62 |

where the acrylamide : bisacrylamide stock was 60 : 0.3% (w/v). Polymerisation of the 30% gel mixture was by addition of 75 µl of 10% (w/v) ammonium persulphate solution and 7.5 µl of TEMED. Neither the 4% or 30% gel mixtures were degassed in order to prevent premature polymerisation during the formation of the gradient. A stacking gel was not used, the plastic comb was inserted directly into the top of the gradient gel and the level topped up with 4% gel mixture.

Once the gel had set the comb was removed and the gel mounted in the electrophoresis tank. The composition of the electrophoresis running buffer was: 380 mM glycine, 50 mM Tris-HCL, pH 8.8. Samples were loaded in a 5% (w/v) sucrose solution with 0.001% (w/v) bromophenol blue. Gels were run at 10 mA constant current overnight and then removed for staining (see Section M4).

Non-denaturing gels were routinely run with molecular weight markers purchased as a kit from Sigma:

| | molecular mass kDa |
|---|---|
| α-Lactalbumin | 14.2 |
| carbonic anhydrase | 29.0 |
| albumin (chicken egg) | 45.0 |
| albumin (bovine) | |
| monomer | 66.0 |
| dimer | 132.0 |
| urease (Jack bean) | |
| dimer | 240.0 |
| tetramer | 480.0 |

## Staining Polyacrylamide-Gels

(A) Coomassie Staining

Resolved proteins were usually visualised by staining with Coomassie brilliant blue R. The gel was immersed in a solution of stain composed of:

| | |
|---|---|
| Methanol | 50% (v/v) |
| Distilled water | 40% (v/v) |
| Acetic acid | 10% (v/v) |
| Coomassie blue | 0.1% (w/v) |

and shaken at room temperature for at least two h. The gel was destained by shaking in two or three changes of destain solution:

| | litres |
|---|---|
| Distilled water | 1.0 |
| Methanol | 0.3 |
| Acetic acid | 0.07 |

at room temperature for at least two h. The gel could be preserved and stored by drying onto a piece of 3 MM Whatman filter paper at 80°C under vacuum. The dried gel was mounted onto a piece of card for support.

(B) Silver Staining

Resolution of very small amounts of protein (< 0.5 μg) was achieved by staining with silver nitrate according to the protocol of Wray et al. (1981). The gel was immersed in three changes of reagent grade methanol

and shaken approximately two h between changes. Immediately prior to staining the gel was immersed and shaken in distilled water for five min. The distilled water was poured away and solution C added:

Solution A: dissolve 0.8 g of silver nitrate in 4 ml of distilled water.

Solution B: mix 1.4 ml of 14.8 M ammonium hydroxide with 21 ml of 0.36% (w/v) sodium hydroxide.

Solution C: add solution A dropwise into solution B with constant stirring and increase volume to 100 ml with distilled water. Solution C must be used within 5 min.

The gel was stained in solution C for 15 min with constant shaking. After staining with solution C the gel was washed in distilled water for 5 min and then developed with solution D:

Solution D: mix 2.5 ml of 1% citric acid and 0.25 ml of 38% formaldehyde and increase the volume to 500 ml with distilled water. Solution D must be fresh.

Protein bands usually appeared after about 15 min constant shaking. Development of the stain was stopped by soaking in 50% methanol. Gels which had been over-stained were cleared by soaking in a 10% (v/v) solution of Kodafix. Gels which were to be stored were first soaked in distilled water for 1-2 h then dried on a piece of 3 MM Whatman filter paper under vacuum at 80°C. Appreciable darkening of the background often occurred at this stage.

(C) Periodic Acid-Schiff (PAS) Staining

The PAS staining protocol used was that of Zacharius (1969) modified after Glossman and Neville (1971). The gel was shaken overnight in fixative:

| | |
|---|---|
| Methanol | 40% (v/v) |
| Acetic acid | 10% (v/v) |

and given a further wash in fresh fixative for one hour the next day. The gel was rinsed for 1-2 min in distilled water immediately prior to staining and then immersed in periodic acid solution (1% (w/v) periodic acid in 7% (v/v) acetic acid) for 50 min with constant shaking in the dark. After a brief wash in water the gel was then shaken in Schiff's reagent for 50 min in the dark. The gel was again washed briefly in distilled water and given three washes of 10 min each in sodium metabisulphite solution (1% (w/v) sodium metabisulphite in 0.1 M hydrochloric acid). After washing in distilled water for one hour the gel could be stored in 7% acetic acid. PAS stained gels invariably fragmented on drying.

## Western Blotting

The Western transfer protocol for electroblotting of proteins onto nitrocellulose filters was based on that of Towbin et al. (1979).

After electrophoresis the gel was soaked in transfer buffer for half an hour:

| | |
|---|---|
| Tris-HCL, pH 8.3 | 25 mM |
| Glycine | 192 mM |
| Methanol | 20% (v/v) |

then layered onto a piece of nitrocellulose filter which had been cut to size and soaked in transfer buffer. The gel and nitrocellulose filter were sandwiched between two pieces of foam and clamped into the gel holder. The gel holder was slotted into the transfer apparatus which had been filled with buffer. The gel was blotted for one and a half hours at 50V.

To check that transfer had occurred efficiently the nitrocellulose filter could be stained with an Amido Black solution:

| | |
|---|---|
| Amido Black | 0.1% (w/v) |
| Isopropanol | 25% (v/v) |
| Acetic acid | 10% (v/v) |

at room temperature by passing the blotted filter once through the stain. Destaining was by gentle agitation in the same solution used to dissolve the Amido Black. For best results the blotted filter was first equilibrated in a solution of destain before staining.

M 6

## Iodination of Protein A

The protocol was providad by Dr. E. A. Jones, University of Warwick.

A column of 1% Sephadex G-50 (medium grade) was poured in a five ml glass pipette and prewashed in 1% BSA made up in PBS. 25 µl of protein A solution (1 mg/ml dissolved in PBS) were mixed with 1 mCi of sodium iodide and 6 µl of chloramine T, 2 mg/ml dissolved in 0.5 M phosphate buffer, at room temperature for 2 min. The volume of the reaction mixture was adjusted to 50 µl by adding the appropriate volume of phosphate buffer to the protein A before the reaction. The volume of the radioactive sodium iodide varied, depending on the batch.

The reaction was stopped by adding:

| | |
|---|---|
| Tyrosine solution | 25 µl |
| 10% BSA solution | 50 µl |
| PBS | 200 µl |

and running the mixture into the Sephadex G-50 column. PBS was used as eluent and 500 µl fractions collected in 1.5 ml eppendorfs. Two peaks of radioactivity were observed, the first corresponding to the iodinated protein A and the second corresponding to free iodine. Iodinated protein A was stored in lead pots at 4°C and used within one month.

Probing Western Blots with Antibodies and Iodinated Protein A

100 µl of rabbit IgG antibodies prepared as described in Section M 16 were added to 30 ml of incubation buffer:

| | |
|---|---|
| BSA | 8% (w/v) |
| Sodium azide | 0.02% (w/v) |

made up in phosphate buffered saline (PBS):

| | |
|---|---|
| $NaH_2PO_4$ | 10 mM |
| NaCl | 150 mM |

and incubated with constant agitation at room temperature overnight. The filter was subsequently washed for one hour in three changes of PBS and then equilibrated with 30 ml of incubation buffer prior to the addition of iodinated protein A.

Five µl of $^{125}I$ with an activity of approximately $2 \times 10^5$ cpm/µl was added to each filter and incubated at room temperature for two h with constant shaking. The iodinated protein A was poured away and the filter washed in 1% (v/v) Triton X-100 made up in PBS until the background of the filter was reduced to between five and ten cpm as measured by a hand-held monitor. The filter was dried using a hair drier and put to film as described in Section M 23.

## Western Blots Probed with Human IgE

The protocol described here was used by Mrs. S. Thorpe at Guy's Hospital Medical School to probe blots of castor bean proteins produced at Warwick.

Nitrocellulose filters carrying the separated castor bean proteins were incubated with 100 µl of pooled serum from ten castor bean sensitive patients, the CB pool, in two ml of PBS containing 0.5% Tween-20 and mixed overnight at room temperature. Unbound serum proteins were washed off with five changes of three ml each of 0.9% saline containing 0.1% Tween-20 and subsequently incubated overnight at room temperature with $^{125}$I-radiolabelled goat anti-IgE. The specific activity of the anti-IgE was 1.3 µCi/µg and two ng were added to two ml of PBS containing 0.5% Tween-20. Unbound, radiolabelled anti-IgE was washed off as before and the paper strips dried and incubated with X-ray film as described in Section M 23.

The protocol for probing filters with IgE required that the filters were no longer than 16 cm and no wider than 1.5 cm. Accordingly, samples to be blotted and probed with IgE were electrophoresed until the bromophenol blue front in the sample buffer was 6 cm from the end of the resolving gel. This migration distance for the bromophenol blue front had previously been determined as equivalent to a migration distance of 15.5 cm for the small subunit of the putative 2S allergen, Li's protein, as defined in Sub-section D 3(C).

Probing Western Blots with Biotinylated Protein A

Biotinylated protein A and streptavidin peroxidase were bought as the Amersham biotin-streptavidin kit. Blotted filters were blocked by incubation in three washes of PBS containing 0.1% (v/v) Tween-20, 0.1% (w/v) BSA. Blocked filters were sealed in plastic bags with 10 ml of blocking buffer to which 100 µl of anti-Li's protein antibodies had been added. The filters were incubated for four h at room temperature with constant, gentle shaking and were then washed five times in 0.1% Tween-PBS for five min per wash.

The filters were again sealed in plastic bags with 10 ml of a 1/300 dilution, in blocking buffer, of biotinylated protein A and incubated for one hour at room temperature. The filters were then washed in five changes of 0.1% Tween-PBS for five min per wash. Finally, the filters were sealed in plastic bags with 10 ml of a 1/300 dilution, in 1% Tween-PBS, of streptavidin peroxidase and incubated at room temperature for half an hour with constant, gentle shaking.

Prior to development the filters were washed twice in 1% Tween-PBS, twice in PBS and once in Enzyme-Linked Immunoabsorbent Assay (ELISA) buffer:

| | |
|---|---|
| Tris-HCL, pH 7.0 | 0.05 M |
| NaCl | 0.9% (w/v) |

each wash lasted for five min. The filters were developed in 100 ml ELISA buffer containing 150 µl hydrogen peroxide and 60 mg of 3,3'-diaminobenzidine. Brown bands usually appeared within 10 min. The filters were dried using a hair-drier and sealed in plastic bags for storage.

## Chemical Cleavage of Proteins Using N-Chlorosuccinimide

The protocol was that of Liachwe and Ochs (1982). The protein to be cleaved was cut out of an SDS gel and washed in two changes of 25 ml of distilled water for 20 min each time. The gel slice was then washed in 10 ml of buffer containing urea, water and acetic acid in the proportions, 1 g/1 ml/1 ml for 20 min with one change. The gel slice was then washed in 5 ml of the same buffer made up to 0.015 M N-Chlorosuccinimide for 30 min. The gel slice was washed in water as before and equilibrated in a buffer containing:

| | |
|---|---|
| Glycerol | 10% (v/v) |
| 2-mercaptoethanol | 15% (v/v) |
| SDS | 3% (w/v) |
| Tris-HCL, pH 6.8 | 0.0625M |

for one and a half hours with three changes. The gel slice was re-electrophoresed as described in Section M 2.

Cleavage of Proteins Using Staphylococcus Aureus V8 Protease

The protocol was that of Bottomley (1982). Proteins were resolved on SDS-PAGE, stained and destained with Coomassie blue as described in Section M 4(A) and the appropriate bands excised. The bands were incubated in digestion buffer:

| | |
|---|---|
| Tris-HCL, pH 6.8 | 125 mM |
| SDS | 0.5% (w/v) |
| Glycerol | 10% (v/v) |
| EDTA | 1 mM |
| Bromophenol blue | 0.001% (w/v) |

until the bromophenol blue no longer turned yellow (2-3 changes) suggesting that the residual acetic acid from the destain had been neutralised. The bands were then incubated in two changes of one ml of digestion buffer for half an hour at 60°C. 10 µl of 0.5 M DTT were added per ml of digestion buffer. The gel slices were then placed, under running buffer (Section M 2), in slots in the stacking gel and 20 µl of Staphylococcus aureus V8 protease solution layered on top. The proteins were run quickly (26 mA, constant current) into the stacking gel. When the bromophenol blue front had reached the interface between the stacking gel and the resolving gel the current was switched off for 7 min to allow limited digestion of the protein. The current was turned on again and the digested protein run into a 15% (w/v) polyacrylamide resolving gel overnight at 22 mA, constant current.

Initially V8 protease was used at three stock concentrations, 0.5, 5.0 and 50.0 µl/ml to determine which concentration gave a useful range

of fragments. Figure R 7(A) shows the results of this standardisation experiment. Stock concentrations of 5.0 and 50.0 μg/ml were found to be effective and were subsequently employed. Stock V8 protease was dissolved in 125 mM Tris-HCL, pH 6.8, 10% (v/v) glycerol and stored at -20°C.

M 12 (A)

Isolation of Protein Bodies and Separation of Water-Soluble and Water-Insoluble Components

The non-aqueous method of Yatsu and Jacks (1968) was followed for the isolation of protein bodies. Castor beans (var. Impala) with the testa removed were ground in glycerol using a mortar and pestle. Three ml of glycerol were added for every gramme of castor beans. The paste was expressed through four layers of muslin and centrifuged at 10,000 rpm for 10 minutes to separate the fat from the protein bodies. The pelleted protein bodies were resuspended in 20 ml of 10 mM Tris-HCL, pH 7.4, containing 1 mM PMSF, 5 mM PCMB, 5 mM 1, 10-phenanthroline and 10 mM EDTA as suggested by Gray (1982) and left on ice for 20 min and then centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was removed and made up to an 80% (w/v) ammonium sulphate concentration. The solution was left stirring on ice for two h and the precipitated proteins pelleted by centrifugation at 15,000 rpm as before.

The insoluble crystalloids were washed twice in 10 mM Tris-HCL, pH 7.4 with a five min centrifugation at 10,000 rpm between each wash. The crystalloids were freeze dried and stored dessicated at 4°C.

In every case samples were centrifuged in an MSE 8 x 50 rotor.

M 12 (B)

Isolation of Water-Soluble and Water-Insoluble Protein from Wheat and Barley Seeds

Ten grammes of barley and ten grammes of wheat were frozen in liquid nitrogen and ground briefly in a coffee grinder. The powder was

suspended in 50 ml of 10 mM Tris-HCl, pH 7.0, containing 1 mM PMSF, 5 mM PCMB, 5 mM 1, 10-phenanthroline and 10 mM EDTA as suggested by Gray (1982) and left on ice for one hour. The protein was expressed through 8 layers of muslin and centrifuged for 10 min. at 10,000 rpm, 4°C, using an MSE 8 x 50 rotor.

The supernatant, containing the water-insoluble protein was poured off and retained. The pellet of protein insoluble in aqueous buffer was dissolved in 10 mM Tris-HCl, pH 7.0, containing 10% (w/v) SDS by heating in a water bath at 65°C for five min.

Purification of a Castor Bean 2S Allergen, Li's Protein

All operations were carried out at 4°C except where otherwise indicated. The protocol was based on that of Li et al. (1977) and the purified protein called Li's protein as defined in Sub-section D 3(C).

Protein bodies of the castor bean endosperm were isolated and the water-soluble protein extracted as described in Section M 12. Sufficient ammonium sulphate to give 80% saturation (51.6 g per 100 ml) was added over a period of one hour on ice with constant, gentle stirring and left for a further hour on ice. The precipitate was collected by centrifugation at 15,000 rpm for 10 min. The pellet was resuspended in five ml of 0.05 M imidazole-HCL, pH 7.0 and extracted once with an equal volume of ether in order to remove remaining fat.

The cleared solution was made up to 5% (w/v) sucrose concentration to increase its density and applied, under buffer, to a column (2.5 x 45 cm) of Sephadex G-50 which had been pre-equilibrated in the same buffer. The column was eluted at 25 ml/h in the same buffer and three ml fractions collected in an LKB Redirac fraction collector. The absorbance at 280 nm of even numbered fractions was measured and an aliquot of each fraction was analysed by SDS-PAGE. A typical elution profile is shown in Figure R 2(A) and the results of SDS-PAGE analysis shown in Figure R 2(B). Peak 2, containing the 2S albumins, was pooled and sufficient solid ammonium sulphate added to give 80% saturation. The protein was precipitated and collected as before, redissolved in five ml of 10 mM Tris-HCL, pH 7.0 and dialysed against five L of the same buffer overnight with one change.

The dialysed 2S albumins were diluted to 10 ml with dialysis buffer and applied to a DEAE-Sephacel column (2.5 x 15 cm) equilibrated in

dialysis buffer and the protein run in at a flow rate of 1.3 ml/min. Unbound proteins were eluted with a column volume of dialysis buffer. The column was eluted with 200 ml of a linear salt gradient (10 mM Tris-HCL, pH 7.0-300 mM NaCl, 10 mM Tris-HCL, pH 7.0). Protein still bound to the column was eluted with a column volume of 500 mM NaCl. Three ml fractions were collected at each stage. The absorbance at 280 nm of even numbered fractions was measured at each stage and aliquots analysed using SDS-PAGE. The conductivity of every tenth fraction was measured. A typical elution profile is shown in Figure R 3(C) and the results of SDS-PAGE analysis shown in Figure R 3(D). The fractions comprising the first peak, fractions 10-20, containing Li's protein, were pooled and sufficient ammonium sulphate added to give 80% saturation. The protein was precipitated, collected and dialysed as before.

Dialysed Li's protein was made up to 10 ml with dialysis buffer and applied to a CM-cellulose column (2.5 x 12 cm) equilibrated in dialysis buffer and run in a flow rate of 0.3 ml/min. One column volume of the same buffer was washed through at a flow rate of 1.5 ml/min and the unbound protein collected. The bound protein was eluted with a linear salt gradient (10 mM Tris-HCL, pH 7.0-300 mM NaCl, 10 mM Tris-HCL, pH 7.0) and the absorbance at 280 nm of even numbered fractions measured at each stage and aliquots analysed using SDS-PAGE. The conductivity of every tenth fraction was measured. A typical elution profile is shown in Figure R 3(E) and the results of SDS-PAGE shown in Figure R 3(F). The protein in the major peak absorbance was precipitated and collected as before. Finally, the protein was dialysed overnight against five L of distilled water with one change, the volume reduced to about two ml using Aquacide and the protein freeze dried.

In every case cebtrifugation was carried out using an MSE 8 x 50 rotor.

## Purification of the Cross Reactive Crystalloid Protein

The crude crystalloid fraction was isolated from 30 g castor beans (var. Impala) as described in Section M 12. All subsequent manipulations were carried out at room temperature to prevent crystallisation of the urea used in the buffer.

Five samples of 50-100 mg of the protein were each resuspended in 200 µl of binding buffer:

| | |
|---|---|
| Tris-HCL | 10 mM |
| Urea | 6 M |
| 2-marcaptoethanol | 5% (v/v) |

at five different pH values (5.0, 6.0, 7.0, 8.0 and 9.0) by incubating at 60°C for 15 min. The samples were added to 500 µl of CM-cellulose slurry pre-swollen in binding buffer and incubated at room temperature for 45 min with constant shaking. The slurry and supernatant fractions were separated by centrifugation for 2 min in a microcentrifuge. The supernatants were removed and the slurries washed three times in 600 µl of binding buffer at the appropriate pH for 10 min per wash. 200 µl of binding buffer containing 0.3 M NaCl, pH 7.0 were added to the slurries and shaken for 20 min. The slurries were pelleted by centrifugation for two min in a microcentrifuge and the supernatants, containing the eluted protein, collected. Samples of the bound and unbound crystalloid proteins at each pH were analysed by SDS-PAGE and a typical result is shown in Figure R 8(A). The pH judged to give optimal binding of the cross reactive crystalloid protein, with the smallest degree of contamination from other proteins, was pH 7.0.

Total crystalloid protein from 15 g of castor beans was dissolved in binding buffer (pH 7.0) as before and applied to a CM-cellulose column (2.5 x 15 cm) pre-washed in five column volumes of binding buffer. The crystalloid protein was run into the column at a flow rate of 0.25 ml/min and unbound protein eluted with 90 ml of binding buffer at a flow rate of 1.5 ml/min. The bound proteins were eluted with a linear salt gradient (0-0.15 M NaCl in binding buffer) at a flow rate of 1.5 ml/min. Protein still bound to the column was eluted with one column volume of 500 mM NaCl in binding buffer. Two ml fractions were collected at each stage. The absorbance at 280 nm of even numbered fractions was measured and aliquots of the fractions were analysed by SDS-PAGE. The conductivity of every tenth fraction was measured. A typical elution profile is shown in Figure R 8(B) and a typical result of SDS-PAGE analysis is shown in Figure R 8(C).

The fractions containing the cross reactive crystalloid protein, 92-108, inclusive, were pooled and dialysed overnight against 5 L of distilled water with one change. In the absence of a high concentration of urea the crystalloid protein precipitated and was collected by centrifugation at 15,000 rpm for 15 min at $4^{o}C$ (MSE 8 x 50 ml rotor).

An attempt was made to establish the conditions required for rigorous purification of the cross reactive crystalloid proteins using chromatofocusing. The chromatofocusing matrix and polybuffer were purchased as a kit from Pharmacia Ltd. The crystalloid protein was resuspended in one ml of starting buffer.

| | |
|---|---|
| Imidazole-HCL, pH 7.4 | 25 mM |
| Urea | 6 M |
| 2-mercaptoethanol | 5% (v/v) |

and dialysed overnight against 300 ml of the same buffer. The protein

was run into a 10 ml column of the anion exchanger, PBE 94, pre-equilibrated in starting buffer, and the column eluted in a 1/8 dilution of polybuffer (pH 4-7) at a flow rate of one ml/min and two ml fractions were collected. The pH of the neat polybuffer had been previously adjusted to pH 4.0 with hydrochloric acid and diluted in 6 M urea but without 2-mercaptoethanol since the possible effects of a strong reducing agent on polybuffer were unknown. The absorbance at 280 nm of even numbered fractions was measured and aliquots of the fractions analysed by SDS-PAGE. An elution profile is shown in Figure R 8(F) and the results of SDS-PAGE analysis are shown in Figure R 8(G). The pH of every tenth fraction was estimated using pH paper and the values are presented in Table R 2.

## Enzymatic Assay of Trypsin and Chymotrypsin Inhibition

The protocol for the trypsin inhibition assay was a gift of Sigma Chemical Co. Ltd. The chymotrypsin inhibition assay was that used by Worthington Biochemicals Ltd as described in their catalogue with slight modifications.

Protein trypsin inhibitors will inhibit the following reaction:

N-Benzoyl-L-Arginine Ethyl Ester (BAEE) -----> N-Benzoyl-L-Arginine + Ethanol.

and chymotrypsin inhibitor will inhibit the following reaction:

N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) -----> N-Benzoyl-L-Tyrosine + Ethanol.

The reaction can be followed spectrophotometrically at 253 nm in the case of BAEE and at 256 nm in the case of BTEE. The stock concentrations of the enzymes and inhibitors were:

| | |
|---|---|
| Trypsin | 1 mg/ml |
| α-Chymotrypsin | 10 mg/ml |
| Lima bean trypsin inhibitor | 1 mg/ml |
| Li's protein | 1 mg/ml |

A range of trypsin and chymotrypsin preparations are available from Sigma Chemical Co., those used in this study are described in the materials section. For the trypsin inhibition assay a range of enzyme-

inhibitor mixes were prepared by combining 0, 10, 20, 30, 40 or 50 µl of inhibitor each with 50 µl of trypsin and making each mixture up to one ml with one mM HCL. The following components were then mixed in a 1 ml quartz cuvette:

| | |
|---|---|
| BAEE (0.248 mM) | one ml |
| Enzyme-inhibitor mix | 33 µl |

and the absorbance of 253 nm measured at 20 sec intervals using a single beam Cecil spectrophotometer at room temperature. The concentration of trypsin was chosen from previous experiments to give linearity for the greatest time period. Trypsin was dissolved in 0.001 M HCL and BAEE was dissolved in 0.67 M sodium dihydrogen orthophosphate, pH 7.6. Both lima bean trypsin inhibitor and Li's protein were dissolved in distilled water.

For the chymotrypsin inhibitory assay 0, 10, 20, 30, 40 or 50 µl of inhibitor were made up to one ml with 80 mM Tris-HCL, pH 7.8, 0.1 M $CaCl_2$. The following components were mixed in a quartz cuvette:

| | |
|---|---|
| BTEE (0.00107 M) | 0.47 ml |
| Chymotrypsin (10 mg/ml) | 1.0 µl |
| Inhibitor Mix | 0.50 ml |

and the absorbance at 256 nm measured in a Cecil single beam spectrophotometer at 20 sec intervals at room temperature. BTEE was dissolved in 50% (w/w) methanol and chymotrypsin in 0.001 M HCL. Both lima bean trypsin inhibitor and Li's protein were dissolved in 0.08 M Tris-HCL, pH 7.8, containing 0.1 M $CaCl_2$.

The ability of lima bean trypsin inhibitor and Li's protein to inhibit both trypsin and chymotrypsin was tested. In each case

absorbance was plotted against time (seconds) for each concentration of inhibitor using the least squares fit method (Montgomery and Swanson, 1969). The gradient of each plot was expressed as a percentage of the gradient of the plot in the absence of inhibitor ($T_o$). These values were plotted against the mass of inhibitor present in each reaction mixture using the least squares fit method. The plot was extrapolated to the x-axis to give the mass of inhibitor required to fully inhibit the standard mass of enzyme present in each reaction mixture.

Inhibitory activity was expressed as the mass of inhibitor required to fully inhibit one mg of enzyme. The molecular masses of lima bean trypsin inhibitor, Li's protein, trypsin and chymotrypsin are: 9 kDa, 11 kDa, 23-8 kDa and 23 kDa, respectively.

## Preparation of Rabbit Anti-Li Protein Antibodies

White lop-eared rabbits were primed with an intramuscular injection in the hind leg of one µg of Li's protein with Complete Freund's Adjuvant. Li's protein had been resolved by SDS-PAGE and electroeluted overnight into a bag of dialysis membrane. Electroelution buffer was that used in SDS-PAGE (see Section M 2) and the electroeluted protein was precipitated with an equal volume of 20% trichloroacetic acid on ice for 30 min.

The injection was repeated twice at intervals of two weeks using Incomplete Freund's Adjuvant. Blood was extracted by cardiac puncture and allowed to clot. The serum was poured off and centrifuged at 15,000 rpm using an MSE 8 x 50 rotor for 10 min at 2 $^{o}$C. Sufficient ammonium sulphate was added to give a concentration of 50% (29.1 g per 100 ml) over a period of half an hour with gentle stirring on ice. The solution was left on ice for one hour and the precipitate collected by centrifugation as before. The precipitate was dissolved in half the original serum volume of PBS (see Section M 7) and dialysed at 4$^{o}$C overnight against five L of PBS with one change. The antibodies were stored as 100 µl aliquots at -20$^{o}$C.

## Extraction of Messenger RNA

No more than 30 g of whole castor beans were frozen in liquid nitrogen and ground in a mortar and pestle. The powder was homogenised in 2-3 volumes (one volume = one ml/g tissue) of extraction buffer:

| | |
|---|---|
| Tris-HCL, pH 9.0 | 50 mM |
| Sodium chloride | 150 mM |
| EDTA | 5 mM |
| SDS | 5% (w/v) |

in a Waring blendor for two min. The homogenate was stirred with an equal volume of 1:1 (v/v) phenol/chloroform for 5-10 min and the phases separated by centrifugation for five min at 5000 rpm at room temperature. The upper aqueous phase was transferred to a beaker and the organic phase re-extracted with a half volume of washing buffer:

| | |
|---|---|
| Tris-HCL, pH 9.0 | 20 mM |
| EDTA | 2 mM |

and the aqueous phases pooled. The pooled aqueous phases were re-extracted with an equal volume of 1:1 (v/v) phenol/chloroform as before. The procedure was repeated twice. The pooled aqueous phases were made up to a sodium chloride concentration of 0.2 M, two volumes of ice cold ethanol added, mixed and allowed to stand overnight at -20°C.

The ethanol precipitate was collected by centrifugation at 10,000 rpm for 15 min at 2°C. The pellets were transferred to corex tubes and resuspended in 3 M sodium acetate pH 5.5 using a glass rod. This

procedure was repeated four times, the pellet collected between each wash by centrifugation at 10,000 rpm for five min. The pellet was finally dissolved in a small volume (0.3 ml per gramme of original tissue) of 0.3 M sodium chloride and precipitated at -20°C overnight after the addition of two volumes of ice cold ethanol.

As before the precipitate was collected by centrifugation at 10,000 rpm for 15 min at 2°C. The pellet was dissolved in 20 ml of oligo dT buffer A:

| | |
|---|---|
| Tris-HCL, pH 7.4 | 20 mM |
| Sodium chloride | 400 mM |
| SDS | 0.2% (w/v) |

and the concentration adjusted so that it did not exceed 100 $A_{260}$ units per ml. The dissolved RNA was added to oligo dT cellulose, pre-swollen in buffer A, at a ratio of 1500 $A_{260}$ units of RNA per 1-2 g of solid and mixed at room temperature for 15 min. The oligo dT cellulose was collected by centrifugation at 5000 rpm for 1-2 minutes and washed three times with buffer A. The oligo dT cellulose was mixed gently, not vortexed, at each wash and collected by centrifugation at 5000 rpm for 2-3 min. The washing step was repeated three times with buffer B:

| | |
|---|---|
| Tris-HCL, pH 7.4 | 20 mM |
| Sodium chloride | 200 mM |
| SDS | 0.1% (w/v) |

and the oligo dT cellulose transferred to a column connected to a Uvicord spectrophotometer and chart recorder. Washing with buffer B was continued until no further absorbance at $A_{260}$ was detectable. Messenger RNA (poly $A^+$ RNA) was eluted with 20 mM Tris-HCL, pH 7.4 pre-heated to

50°C. The eluted poly $A^+$ RNA was made up to a sodium chloride concentration of 0.2 M and precipitated at -20°C overnight on addition of two volumes of ice cold ethanol.

The precipitate was collected by centrifugation at 15,000 rpm for 20 min at 4°C, washed twice in 70% ethanol and stored in 10 mM Tris-HCL, pH 7.4 at -80°C.

In every case centrifugation was performed using an MSE 8 x 50 rotor.

## In vitro Translations and Immunopracipitations

Rabbit reticulocyte lysate kits were obtained from Amersham UK Ltd., and used according to the manufacturers instructions. Reactions were incubated in 25 µl volumes at 37°C for 40 min and protein synthesis was assayed by uptake of ($^{35}$S) methionine into hot TCA-insoluble material, samples being processed by the method of Mans and Novelli (Maniatis, 1982) and counted in Beckman commercially supplied scintillant.

Samples for immunopracipitation were mixed with an equal volume of:

| | |
|---|---|
| Nonidet P-40 | 1% (v/v) |
| Tris-HCL, pH 7.4 | 20 mM |
| EDTA | 2 mM |
| Sodium chloride | 150 mM |
| PMSF | 200 mM |

After a 45 min incubation at room temperature insoluble material was pelleted in a Beckman airfuge at 20 psi for 5 min and 2 µl of null serum was added to the supernatant. After 15 min at room temperature 30 µl of protein A-Sepharose was added and the mixture was reincubated at room temperature for 30 min. The beads were pelleted and 2.5 µl of anti-L1's protein antibodies (Section M 16) added to the supernatant. After one hour at room temperature 50 µl of protein A-Sepharose was added and the mixture was incubated at room temperature for 30 min. The beads were then pelleted and washed three times with:

| | |
|---|---|
| Tris-HCL, pH 7.4 | 20 mM |
| Sodium chloride | 150 mM |
| EDTA | 1 mM |
| NP40 | 0.2% (v/v) |

then twice in the same buffer but containing 500 mM sodium chloride, and once in 10 mM Tris-HCL, pH 7.4. In the case of samples to be reduced the beads were resuspended in 40 µl of 0.5 M DTT was added and the sample boiled for three minutes. Unreduced samples were treated with one M acetic acid to liberate the bound immunoprecipitate. Material eluted from the beads was analysed using SDS-PAGE as described in Section M2.

## Fluorography

The method was based on that of Bonner and Laskey (1974). After electrophoresis the gel was washed with two changes of dimethylsulphoxide and immersed for three h in a solution containing 2,5-diphenyloxazole (22%, w/v) in dimethylsulphoxide. The gel was then soaked in circulating tap water for half an hour and dried.

## cDNA Synthesis

### (A) Growth and Harvesting of Castor Bean Seeds

Castor bean plants (Ricinus communis) were grown from seed in John Innes No. 1 compost in a greenhouse. They were maintained at 20°C and illuminated with sodium lamps (10,000-12,000 lm $m^{-2}$) with a light : dark regime of 16 h : 8 h (Lamb, 1984).

The development of castor bean plant seeds has been divided into seven stages based on size, formation of the testa and state of hydration (Roberts and Lord, 1981). Seeds were harvested at stages D-E for cDNA cloning because at this stage the 2S albumin precursor is most abundant.

### (B) cDNA Synthesis Using the S1 Nuclease Method

The protocol was provided by Dr. L. M. Roberts at the University of Warwick. Messenger RNA was reverse transcribed (Buell et al., 1978; Retzel et al., 1980) at 50 ng/µl in the presence of:

| | µl | |
|---|---|---|
| Reverse transcriptase mix | 12.0 | |
| Distilled water | 0.9 | |
| Poly $A^+$ | 5.0 | |
| $\alpha^{32}$P dGTP (~3000 Ci/mmol) | 1.0 | (1 µCi) |
| Reverse Transcriptase [25 units] | 1.1 | |
| | 20 | |

where reverse transcriptase mix contains:

| | |
|---|---|
| Tris-HCL, pH 8.3 | 100 mM |
| Magnesium chloride | 17 mM |
| Potassium chloride | 166 mM |
| dATP, dCTP, dTTP | 1.7 mM |
| dGTP | 0.8 mM |
| Oligo dT | 0.1 mg/ml |
| DTT | 17 mM |
| Distilled water | 162 µl |
| | 240 µl |

the reaction mixture was incubated for 45 min at 42°C. After 45 min another 1 µl of reverse transcriptase was added along with 19 µl of dilution mix:

| | |
|---|---|
| Tris-HCL, pH 8.3 | 5 mM |
| DTT | 5 mM |
| dGTP | 2.5 mM |

and the reaction mixture reincubated for 45 min at 45°C. Aliquots were removed for analysis by agarose gel electrophoresis (Section M 21) and estimation of the synthetic yield (Sub-section M 20(E)).

The mRNA-cDNA hybrids were denatured by boiling for three min and cooling rapidly on ice. The following reagents were then added:

| | µl |
|---|---|
| Nucleotide mix | 7.6 |
| Reverse Transcriptase Mix | 36.0 |
| DNA polymerase I (20 u) | 1.0 |
| $\alpha^{32}$P dGTP | 1.0 |
| | 45.0 |

where nucleotide mix is composed of:

dATP 0.67 mM; dCTP 0.67 mM; dGTP 0.67 mM; dTTP 0.67mM;
HEPES-KOH, pH 6.9 0.67 M; KCl 0.25 M

and the reaction mixture incubated for a further six h at 22°C. The reaction mixture was spun briefly in a micro-centrifuge after 2.5 and 4.5 hours to prevent condensation accumulating on the eppendorf walls. Aliquots were removed for analysis by alkaline agarose gel electrophoresis (Section M 21) and for estimation of the synthetic yield (Sub-section M20 (E)). The reaction was stopped by addition of 10 µl of 0.25 M ETDA and was phenol extracted twice and precipitated at -20°C as decribed in Section M 22.

The precipitate was collected by centrifugation in a microcentrifuge for five min at 4°C, washed twice in 70% ethanol, dessicated and resuspended in 40 µl of distilled water. The cDNA was re-precipitated as before, left in dry ice for one hour, and collected and washed as described. The re-precipitated cDNA was dissolved in 10 µl of distilled water and added to:

| | µl |
|---|---|
| S1 Nuclease buffer | 2.0 |
| Distilled water | 7.0 |
| S1 Nuclease (120 units) | 1.0 |
| | 20 |

where S1 nuclease buffer is composed of:

| | |
|---|---|
| Sodium chloride | 3 M |
| Sodium acetate | 0.3 M |
| Zinc chloride | 30 mM |

The reaction mixture was incubated for 15 min at 37°C and then for 15 min at 15°C. The reaction was stopped by the addition of one µl of 0.25 M EDTA, an aliquot removed for analysis on alkaline agarose gel electrophoresis and the remainder phenol extracted. The S1-treated cDNA was precipitated, resuspended in 10 µl of distilled water and stored at -20°C.

(C) cDNA Synthesis Using the RNase H Method

The RNase H protocol was a gift of Dr. A. Colman, University of Warwick and was based on the method of Gubler and Hoffman (1983). One µg of poly $(A)^+$ RNA was added to a final volume of 50 µl of the following reaction buffer:

| | |
|---|---|
| Tris-HCL, pH 8.3 | 50 mM |
| KCL | 50 mM |
| $MgCl_2$ | 10 mM |
| Oligo dT | 100 µl/ml |
| DTT | 10 mM |
| dATP | 1 mM |
| dCTP | 1 mM |
| dTTP | 1 mM |
| dGTP | 0.5 mM |
| $^{32}P$ dGTP | 30 µCi |
| Sodium pyrophosphate | 4 mM |
| Placental RNAsein | 20 units |

Three μl (52 units) of reverse transcriptase were added and the reaction mixture incubated at 42°C for one hour. At the end of the reaction time 2.5 μl of 0.5 M EDTA were added, the mixture phenol extracted and precipitated on dry ice.

The precipitated RNA-DNA hybrid was resuspended in a final volume of the following reaction buffer:

| | |
|---|---|
| 10 x Reaction Buffer | 1 x |
| BSA | 50 μg/ml |
| 40 uM dCTP | 40 μM |
| dTTP | 40 μM |
| dGTP | 40 μM |
| $^{32}$P dGTP | 30 μCi |

where 10 x reaction buffer contains:

| | |
|---|---|
| Tris-HCL, pH 7.5 | 200 mM |
| $MgCl_2$ | 50 mM |
| Ammonium sulphate | 100 mM |
| KCL | 1 M |

30 units (three μl) of DNA polymerase 1 and three units (1.5 μl) of RNAse H were added and the reaction mix incubated for one hour at 12°C and then for one hour at 22°C. The reaction was stopped by the addition of four μl of 0.5 M EDTA and the mixture phenol extracted.

First and second strand syntheses were followed by alkaline agarose gel electrophoresis (Section M 21) and the synthetic yield calculated as described in Sub-section M 20(E).

(D) Assay for the Incorporation of Radiolabelled Nucleotides During cDNA Synthesis

At the end of both first and second strand syntheses duplicate one µl aliquots were removed and added to 200 µl of ice cold 0.2 M sodium phosphate buffer, pH 7.0. 20 µl of the buffer containing the reaction sample were removed and spotted onto a Whatman GF/C filter and left to dry at room temperature. This aliquot provided a measure of the total amount of radioactivity in the reaction mixture. One ml of ice cold TCA was added to the remaining 180 µl, mixed and left to precipitate on ice for half an hour with 10 µgs of single stranded salmon sperm DNA as carrier. The mixture was filtered through a GF/C filter and washed twice with three mls of ice cold 10% TCA and once with one ml of ethanol. The filter was dried with a hair drier, suspended in Beckman non-aqueous scintillant and the precipitated, bound radioactivity counted.

(E) Calculating the Synthetic Yield of cDNA

The amount of radiolabelled nucleotide incorporated was assayed by the method described in Sub-section M 20(D) and expressed as a percentage of the total radioactivity in the reaction mixture. The mass of cDNA synthesized was derived from the relationship:

$$P \times N \times 340 \times 4$$

where:

P = counts incorporated expressed as a percentage of the total radioactivity in the reaction mixture.

N = the number of moles of unlabelled nucleotide in the reaction mixture.

340 - the gramme formula mass of a single nucleotide.

4 - the number of different nucleotides in the reaction mixture.

The synthetic yield was the mass of cDNA synthesised, expressed as a percentage of the mass of the template.

## Alkaline Agarose Gels

cDNA synthesis was followed on 1.2% denaturing agarose gels (McDonnell et al., 1977) made up in running buffer; 30 mM NaOH, 2 mM EDTA. The samples were loaded in running buffer made up to 0.4% (w/v) bromophenol blue. Gels were run at 90 mA overnight and dried at 80°C under vacuum prior to autoradiography.

## Phenol Extraction and Precipitation of Nucleic Acids

The volume of the nucleic acid solution was increased to at least 50 µl with distilled water and a half volume of 1:1 (v/v) phenol : chloroform added and mixed by rapid pipetting. The mixture was centrifuged for two min in a microcentrifuge and the aqueous phase removed and re-extracted. The whole procedure was repeated. Finally the aqueous phase was extracted with an equal volume of ether.

Phenol extracted samples were precipitated by addition of a half volume of 7 M ammonium acetate and two volumes of ethanol, both having been chilled to -20°C. Precipitation was either overnight at -20°C or for one hour in dry ice.

## Autoradiography

Dried fluorograms containing polypeptides labelled with ($^{35}$S)-methionine were exposed to X-Omat S (Kodak) X-ray film at -70°C. The exposure time depended on the amount of label in the gel but was typically between 7-14 days. Dried agarose gels containing nucleic acids labelled with $^{32}$P were exposed to X-Omat S (Kodak) X-ray film at -70°C. The exposure time was typically between 12 and 48 h.

Nitrocellulose filters probed with $^{125}$I-protein A were exposed to X-Omat S (Kodak) X-ray film usually for two to three days at room temperature.

## Photography

Developed X-ray films and stained protein gels were viewed on a light box and photographed using 35 mm Panatomic-X film (Kodak) and an orange filter in the case of protein gels stained with Coomassie blue. The film was developed for 10 min with Acutol (Paterson) and fixed for five min with Kodafix. The negatives were washed thoroughly with tap water before drying. Kodabrom paper of the appropriate grain size (F1-F4 range) was used to make prints. The paper was developed using Ilford Contrast FF and fixed with Kodafix.

# CHAPTER 3

# RESULTS AND DISCUSSION

SECTION 1

# Estimation of the Number of Putative Castor Bean Allergens.

Summary of Results

At least four proteins were identified as the major IgE-binding species on a Western blot of castor bean protein probed with pooled sera from ten patients allergic to castor beans.

The reactive proteins appeared as a doublet composed of two proteins of molecular masses 48 kDa and 50 kDa, a single protein of molecular mass 34 kDa and one or more proteins of molecular mass approximately 10 kDa.

Figure R 1 Western blot of castor bean protein probed with pooled sera from castor bean-sensitive patients

Protein was extracted from the castor bean endosperm by grinding 10 g of endosperm tissue in 10 mM Tris-HCl, pH 7.0, 10% (w/v) SDS in a Waring Blendor for two minutes. The homogenate was strained through 8 layers of muslin and the solubilised protein retained.

Approximately 20 µg of protein were reduced with dithiothreitol and resolved by SDS-PAGE using a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel (Section M 2) and blotted onto nitrocellulose (Section M 5). The blot was probed with 100 µl of a serum pool derived from 10 patients allergic to castor beans, the CB pool, and then with iodinated anti-IgE antibodies as described in Section M 8. The filter was exposed to X-ray film for 21 days (Section M 23).

The molecular weight markers were those described in Section M 2 for use in SDS-PAGE. The molecular weight markers were resolved on the same gel but were blotted onto a separate nitrocellulose filter and stained with Amido Black.


kDa
50
34
10

## Strategy for the Preliminary Identification of Castor Bean Protein Allergens.

The strategy adopted for the preliminary identification of allergenic castor bean proteins was to probe Western blots of crude mixtures and then purified preparations of castor bean proteins with the CB pool, pooled sera from ten patients known to give a positive skin test when challenged with castor bean extract. In this way a qualitative measure of the IgE response against individual castor bean proteins was obtained.

The use of pooled sera from a number of hypersensitive patients was intended to ensure the identification of major IgE-binding proteins; major in the sense originally defined by Lowenstein [1978] as "antigens that bind IgE from at least 50% of the sera of patients tested and demonstrate strong binding to at least 50% of these".

The ability of individual castor bean proteins on a Western blot to bind IgE from the CB pool constituted the sole criterion of allergenicity in this study and, as such, is open to a number of criticisms. No attempt was made to correlate IgE-binding ability with biological activity for the practical reason that it would require a purified preparation of the putative allergen to do so. In the case of purified dust-mite allergens it has been shown that although the proteins reacted strongly with IgE in a RAST they were only weakly active in skin prick tests [Krilis et al., 1984]. These results suggest that there are factors other than the level of specific IgE involved in eliciting an allergic response. In support of this view it has been shown that basophils from up to 60% of normal individuals cannot be passively sensitised when incubated with serum from allergic subjects

even though the basophils had bound IgE [Geha, 1984].

In general, levels of specific IgE have been shown to correlate positively with biological activity as assayed by skin and nasal challenge tests and by *in vitro* basophil or mast cell histamine release [Stenius *et al*., 1971; Hogarth-Scott *et al*., 1973; Norman *et al*., 1973]. A disparity between the level of specific IgE and the biological activity of the protein, are only of importance from the clinical viewpoint; a major IgE-binding protein which shows little biological activity is still of interest as a member of the small group of proteins known to be capable of eliciting a strong IgE response.

In the case of a complex mixture of proteins such as that presented in Figure R 1, the amount of any individual protein is unknown and may be small enough to be limiting. The amount of IgE bound by a protein may not, therefore, reflect the true proportion of IgE specific for that protein in the sera. This problem can be overcome, to an extent, by loading as much protein onto the gel as possible without compromising resolution.

It is worth emphasising that the unequivocal identification of an allergen requires that subjects sensitised to the source of the allergen show both an elevated level of allergen-specific IgE and a hypersensitive response on being challenged with the allergen [Platts-Mills, 1975]. A highly purified preparation of a putative allergen is required, therefore, before these criteria can be shown to have been fulfilled.

Estimation of the Number of Major Castor Bean Allergens.

Figure R 1 shows that a range of castor bean proteins react with IgE from the CB pool. The major IgE-binding proteins are judged to be a doublet of proteins of molecular masses 50 kDa and 48 kDa, a single protein of molecular mass 34 kDa and one or more proteins of molecular mass about 10 kDa which bind most IgE. Since the proteins were reduced prior to SDS-PAGE the higher molecular weight proteins are not multimers of the lower molecular weight proteins stabilised by disulphide bonds. There are, therefore, at least four major protein allergens as determined by their ability to bind IgE from the CB pool. On the basis of its size the 10 kDa protein, or proteins, may correspond to the 2S storage protein allergen proposed by Youle and Huang [1978b] discussed in the Introduction, Sub-section I 3(D).

## Conclusions

Sera from patients allergic to castor bean have elevated levels of IgE specific for at least four reduced castor bean proteins. These proteins may be the major allergens of castor beans.

SECTION 2

# Identification of the 10 kDa Putative Allergen as a Component of the 2S Storage Albumin Fraction.

Summary of Results.

Water-soluble storage proteins were fractionated by gel filtration yielding a fraction enriched for the 7S lectins and a fraction enriched for the 2S albumins.

Western blot analysis identified the 10 kDa putative allergen as a reduced component of the 2S storage albumin fraction. A group of unreduced 2S storage albumins reacted strongly with the CB pool. The most reactive unreduced component was a doublet of molecular mass about 12 kDa.

The 7S lectin fraction did not bind IgE from the CB pool.

Prior Evidence to Suggest That the 2S Albumins are the Major Castor Bean Allergens.

The storage proteins of the castor bean are abundant and well characterised [see Introduction, Section 4]. They can be divided into three groups mainly on the basis of size and solubility; the water-soluble 7S lectins and 2S albumins and the water-insoluble 11S crystalloids. Youle and Huang [1978b] had previously suggested that the 2S albumins comprised the major allergenic fraction, CB-1A, characterised by Spies and colleagues in the 1940's [for a review see Berrens, 1971]. They compared various physicochemical properties of the 2S albumins and the CB-1A fraction and found them to be very similar. IgG antibodies raised against CB-1A precipitated the 2S albumins. It was concluded that the CB-1A fraction was composed of the 2S albumins. To test this conclusion and to more accurately identify the putative allergens shown in Figure R 1 the water-soluble storage proteins were separated into 2S albumins and 7S lectins and each probed with the CB pool.

Figure R 2(A) Elution profile of water-soluble castor bean protein resolved by gel filtration

Water-soluble castor bean protein was isolated as described in Sub-section M 12(A) and applied to a column (2.5 x 45 cm) of Saphadex G-50 under the conditions described in Section M 13. Three ml fractions were collected and the absorbance at 280 nm of aliquots of the even numbered fractions was measured and plotted against fraction number.

A 280
0.5
1.0
1.5
2.0
2.5
0
10
20
30
40
50
60
Fraction Number
1
2

Figure R 2(B) SDS-PAGE analysis of the absorbance peaks shown in Figure R 2(A)

The protein in peaks one and two (Figure R 2(A)) was precipitated, collected and dialysed as described in Section M 13. Aliquots of peak one (five $\mu$l) and peak two (eight $\mu$l) were analysed by SDS-PAGE using a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel (Section M 2). Track:

| | |
|---|---|
| T | unfractionated soluble protein, unreduced |
| 1 | peak 1 (lectins), unreduced |
| 2 | peak 2 (2S albumins), unreduced |
| M | molecular weight markers, unreduced |

The molecular weight markers were those described in Section M 2 for use in SDS-PAGE.

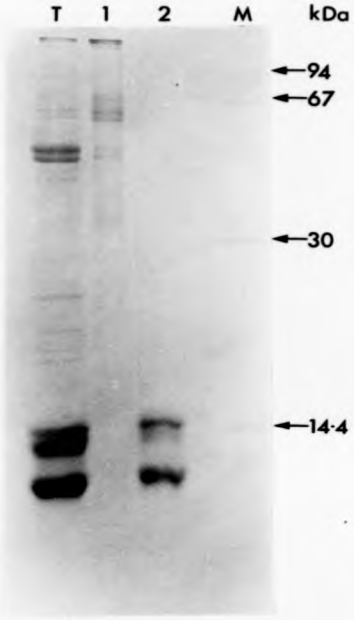

T
1
2
M
kDa
94
67
30
14·4

Figure R 2(C) The reduced and unreduced 2S albumins probed with the CB pool

Duplicate aliquots of eight µl (five µg) of the 2S albumins were resolved by SDS-PAGE (Section M 2) in the presence and in the absence of a reducing agent, dithiothreitol, and stained with Coomassie blue (Sub-section M 4(A)), Figure R 2(C) Part A:

Track 1 unreduced 2S albumins

Track 2 reduced 2S albumins

An identical gel was blotted onto nitrocellulose (Section M 5), probed with the CB pool (Section M 8) and exposed to film for eight days (Section M 23), Figure R 2(C) Part B.

The 2S albumins stained with Coomassie blue were electrophoresed over a distance 4.5 cm longer than those blotted and probed with the CB pool in accordance with the IgE blotting protocol (Section M 8).

In both cases a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel was used.

The arrow in Figure R 2(C), Part A indicates the reduced 2S albumin which reacts with the CB pool.

Figure R 2(C) <u>The reduced and unreduced 2S albumins probed with the CB pool</u>

Duplicate aliquots of eight µl (five µg) of the 2S albumins were resolved by SDS-PAGE (Section M 2) in the presence and in the absence of a reducing agent, dithiothreitol, and stained with Coomassie blue (Sub-section M 4(A)), Figure R 2(C) Part A:

| | |
|---|---|
| Track 1 | unreduced 2S albumins |
| Track 2 | reduced 2S albumins |

An identical gel was blotted onto nitrocellulose (Section M 5), probed with the CB pool (Section M 8) and exposed to film for eight days (Section M 23), Figure R 2(C) Part B.

The 2S albumins stained with Coomassie blue were electrophoresed over a distance 4.5 cm longer than those blotted and probed with the CB pool in accordance with the IgE blotting protocol (Section M 8).

In both cases a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel was used.

The arrow in Figure R 2(C), Part A indicates the reduced 2S albumin which reacts with the CB pool.

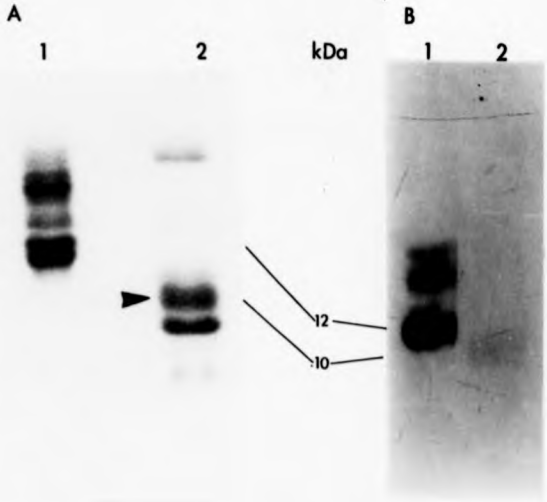
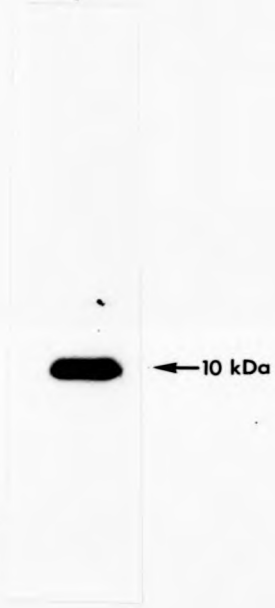

A
1
2
kDa
B
1
2
12
10

Figure R 2(D) Western blot of the reduced 2S albumins probed with the CB pool

Two μg of the reduced 2S albumins were resolved by SDS-PAGE (Section M 2) using a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel. The proteins were blotted onto nitrocellulose (Section M 5), probed with the CB pool (Section M 8) and exposed to film for 24 days (Section M 23).


1
10 kDa

D 2(C)

Western Blot Analysis of the 2S Albumins and 7S Lectins Probed with the CB Pool.

A partial purification of the 7S lectins and 2S albumins was achieved by gel filtration through Sephadex-G 50 [Figures R 2(A) and R 2(B). Probing the reduced 2S albumins with the CB pool [Figure R 2(C), Part B, track 2] showed a single, weakly reactive protein which corresponded to the 10 kDa protein in Figure R 1 and is one of a group of reduced 2S albumins [indicated by an arrow in Figure R 2(C), Part A, track 2].

Compared to the 10 kDa protein shown in Figure R 1 the 10 kDa protein shown in Figure R 2(C), Part B, reacted weakly with IgE from the CB pool. The low intensity of the 10 kDa band is partly due to the fact that the filter shown in Figure R 2(C), Part B, had only been exposed to film for 8 days, compared to 21 days in the case of the filter shown in Figure R 1. In addition, considerable variation was reported in the efficiency with which identical immunoblots bound IgE from the CB pool. A second filter carrying reduced 2S albumin protein was probed with the CB pool and exposed to film for 24 days (Figure R 2(D)). The reactive 10 kDa protein is clearly visible.

Comparing track 1 of Figure R 2(C), Part B with track 1 of Figure R 2 (C), Part A, showing the unreduced 2S albumin fraction stained with Coomassie Blue, the lowest molecular mass doublet, of about 12 kDa, clearly reacts most strongly with the CB pool. Some of the higher molecular mass proteins also reacted quite strongly with the CB pool. These observations support the conclusion of Youle and Huang that the 2S albumins are the major castor bean allergens and demonstrate that the unreduced 2S albumins react more strongly with the CB pool than the reduced 2S albumins.

Track 1 of Figure R 2(C) Part B, the blot of unreduced 2S albumins, could not be compared with a blot of total unreduced castor bean protein showing unreduced protein which reacted with the CB pool since repeated attempts failed to generate an example of the latter. That a range of unreduced 2S albumin proteins should react strongly with the CB pool whereas only a single reactive band of the reduced 2S albumin fraction is seen may suggest that the antigenicity of some of the unreduced 2S albumins entirely depends on the integrity of their three-dimensional structure. Alternatively, the reactive 2S albumins may share a common, allergenic subunit; the 10 kDa IgE-binding protein of the reduced 2S albumin fraction.

Both the unreduced and reduced 7S lectins failed to bind IgE from the CB pool, a result which was reproducible.

## Conclusions

The 10 kDa putative allergen identified in Figure R 1 is a reduced component of the 2S albumin fraction and is probably a single protein.

Most of the proteins in the unreduced 2S albumin fraction reacted with the CB pool. The major IgE-binding protein of the unreduced 2S albumin fraction has an apparent molecular mass of 12 kDa. The 10 kDa putative allergen may be a subunit common to all the reactive, unreduced 2S albumins.

## SECTION 3

# Purification of the Major IgE-Binding Protein From the Unreduced 2S Storage Albumin Fraction.

Summary of Results.

A castor bean 2S storage albumin had previously been purified and sequenced (Li et al., 1977; Sharief and Li, 1982) and had been shown to consist of a large and a small subunit. The purified 2S albumin was compared with the 2S albumin fraction (described in the previous section) using SDS-PAGE and corresponded to the major IgE-binding protein.

The purified 2S albumin was not homogeneous but consisted of two proteins of molecular mass about 12 kDa under non-reducing conditions on an SDS-polyacrylamide gel. The purified large subunit also consisted of at least two proteins.

A protocol for the purification of the major IgE-binding protein was developed from that used by Li et al. (1977) to purify the 2S albumin.

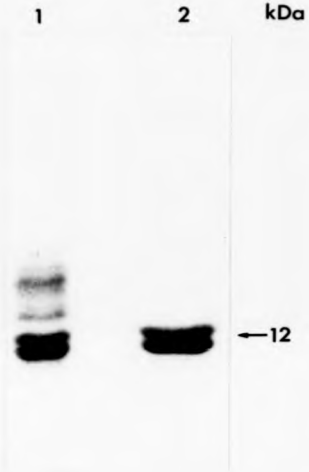
It was found that the major IgE-binding protein could not be silver stained.

Figure R 3(A) Comparison of the 2S albumins with the castor bean protein isolated by S.S.Li. Li's protein

Four μg of the castor bean 2S albumin purified by Li et al. (1977), a gift from Dr. S. S. Li, and 6 μg of the 2S albumin fraction shown in Figure R 2(B), Track 2, were resolved and compared by SDS-PAGE using a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel (Section M 2). Track:

| | |
|---|---|
| 1 | 2S albumins, unreduced |
| 2 | Li's protein, unreduced |

The proteins were stained with Coomassie blue (Sub-section M 4(A)).


1
2
kDa
12

Identity of a 2S Storage Albumin Previously Purified and Sequenced.

A 2S storage albumin from castor beans had previously been purified [Li et al., 1977] and sequenced [Sharief and Li, 1982]. The protein was composed of two subunits and had sequence homology with a Bowman-Birk serine protease inhibitor from lima bean [Tan and Stevens, 1971]. The amino acid composition was similar to that of the allergenic CB-1A fraction of castor beans [Li et al., 1977]. For convenience this protein will be referred to as Li's protein.

A sample of Li's protein, provided by Dr. Steven Li, was compared with the 2S albumin fraction described in the previous section [Figure R 2(B), track 2] using SDS-PAGE. The unreduced protein appeared as a doublet corresponding to the low molecular mass doublet of the unreduced 2S albumin fraction [Figure R 3(A), track 2]. This doublet is the major IgE-binding species of the unreduced 2S albumin fraction [see Track 1, Figure R 2(C), Part B].

The purified subunits of Li's protein are shown in Figure R 3(B). The large subunit is composed of two major polypeptides which also contaminate the small subunit preparation. The small subunit appears as a faint smear and either binds Coomassie blue inefficiently or, because of its small size, is leached from the gel.

Except for the highest molecular mass protein of the reduced 2S fraction the combined large and small subunit polypeptide pattern is clearly identical to that of the reduced 2S albumin fraction [Figure R 2(C), Part A, track 2]. This observation suggests that most, if not all, of the 2S albumins are composed of the same subunits and extends the suggestion in the previous section that most of the 2S albumins have one subunit in common, the allergenic 10 kDa protein of the reduced 2S albumin fraction.

Figure R 3(B) SDS-PAGE analysis of the subunits of Li's protein

Five μg each of unreduced Li's protein, the purified large subunit and the purified small subunit, gifts from Dr. S. S. Li, were analysed by SDS-PAGE using a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel (Section M 2):

| | |
|---|---|
| Track 1 | unreduced Li's protein |
| Track 2 | large subunit |
| Track 3 | small subunit |

The proteins were stained with Coomassie blue (Sub-section M 4(A)).


1
2
3

The Heterogeneity of Li's Protein and its Large Subunit.

The observation that Li's protein appears as a doublet on a non-reducing, denaturing polyacrylamide gel has a number of possible explanations. The doublet may be composed of two different proteins; this possibility can be discounted since the sequence is homogeneous except for one position at which both serine and leucine were found [see Figure I 1]. Such a small degree of sequence heterogeneity also cannot account for the apparent molecular weight difference between the two bands.

The protein may be glycosylated, the higher molecular weight band being more glycosylated than the lower. Periodic Acid-Schiff staining [PAS staining] of Li's protein suggests that it is not glycosylated since it failed to stain. The data is not shown since the poor contrast between the positive control band, ovalbumin, and the background made photography impossible. Tully and Beevers (1976) identified two major castor bean albumins of molecular masses 10.3 kDa and 12.5 kDa. Neither of these proteins were glycosylated.

Data presented in Section 9 of the Results and Discussion Chapter identifies the precursor to Li's protein. The precursor had previously been characterised by Roberts and Lord [1981b] in their study of the biosynthesis of the castor bean agglutinin and it is known to be unglycosylated. Further, the sequence of Li's protein suggests that it may be a serine protease inhibitor similar to those of the Bowman-Birk class, a suggestion confirmed by data presented in Section 5 of the Results and Discussion Chapter. Bowman-Birk inhibitors are not glycosylated. Finally there is now good evidence to suggest that an exposed asparagine-x-serine/threonine sequence, where x is any amino

acid, is the main site of glycosylation in proteins [Fleet and Lennarz, 1977; Hart et al., 1979]. This sequence is not found in Li's protein. Taken in concert the evidence strongly suggests that Li's protein is not glycosylated.

It may be that the lower band of Li's protein doublet is a degradation product of the upper band, degradation proceeding as the protein migrates through the gel. The equivalent amounts, as judged by eye, of both upper and lower bands argues against this idea.

The equal intensity of the upper and lower bands, together with the high cysteine content of Li's protein, suggests a possible and novel explanation; that Li's protein is capable of adopting, with equal likelihood, alternative conformations. Assuming these two conformations to be dependent on disulphide bonding then a non-reducing, SDS-polyacrylamide gel would not completely denature the two structures. The two conformations could be fixed at the time of disulphide bond formation in the endoplasmic reticulum, in which case the two forms would have different disulphide bond patterns. Alternatively, the disulphide bond pattern could be the same in both cases but the basic structure may exist in one of two conformations in equilibrium with each other. In both models the alternative conformations are assumed to be of equal stability. The same arguments would apply to the purified large subunit which shows two clearly separated bands on SDS-PAGE [Figure R 3(B), track 2]. In this respect it is noteworthy that six out of the eight cysteine residues of Li's protein are located in the large subunit [Figure I 1]. A more dramatic example of the possible effect of disulphide bonding on conformation and electrophoretic mobility is the behaviour on SDS-polyacrylamide gel of the precursor to Li's protein in the presence and absence of dithiothreitol [Figure R 9(A), tracks 5 and 6].

Probably the most credible explanation, though, is that there are two genes encoding Li's protein and that the translation product of one is truncated at the carboxyl terminus. The molecular weight difference apparently resides in the large subunit which appears as two polypeptides [Figure R 3(B), track 2] so it is probable that the truncation affects this subunit. Since only a single precursor has been identified for Li's protein [see Section 9, Results and Discussion Chapter] it is likely that the large and small subunits are encoded by a single gene of which there may be two variants. It is well established that many seed storage proteins are encoded by multigene families [for example see Casey and Domoney, 1984]. Further, four isoinhibitors of the soybean Bowman-Birk protease inhibitor are known to be almost identical except for the length of their amino terminii [Huang et al., 1977; Odani and Ikenaka, 1978].

## Terminology: The Definition of Li's Protein

The remainder of this section will deal principally with the purification of the major IgE-binding protein of the 2S storage albumin fraction which corresponds to the castor bean 2S albumin isolated by Li *et al*. [1977]. The protein purified by Li has been referred to as Li's protein.

Unless otherwise stated the name Li's protein will subsequently be taken to mean the protein purified in this study and not the equivalent protein supplied by Dr. Steven Li.

Figure R 3(C) Elution profile of the 2S albumins fractionated on a DEAE-Sephacel column

2S albumins isolated from 60 g of castor beans were applied to a DEAE-Sephacel column (2.5 x 15 cm) and bound protein eluted with a 0-0.15 M NaCl gradient as described in Section M 13. The arrows in Figure R 3(C) indicate the beginning and end of the salt gradient. Protein still bound to the column was eluted with one column volume of 0.5 M NaCl.

Two ml fractions of both bound and unbound protein were collected and the absorbance at 280 nm of the even numbered fractions measured and plotted against fraction number. The conductivity of every tenth fraction was measured and the corresponding sodium chloride concentration plotted against fraction number.

A280 (·)
Fraction Number
NaCl (mM) -o-
1
2
3

Figure R 3(D) SDS-PAGE analysis of the absorbance peaks shown in Figure R 3(C)

The protein in peaks 1, 2 and 3 (Figure R 3(C)) was precipitated, collected and dialysed separately as described in Section M 13. Five µl aliquots of peak 1, peak 2 and peak 3 were analysed by SDS-PAGE along with four µg of unfractionated 2S albumins:

| | |
|---|---|
| Track T | unfractionated 2S albumins, unreduced |
| Track 1 | peak 1, unreduced |
| Track 2 | peak 2, unreduced |
| Track 3 | peak 3, unreduced |
| Track M | molecular weight markers, reduced |

The molecular weight markers were those described in Section M 2 for use in SDS-PAGE. A 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel was used.

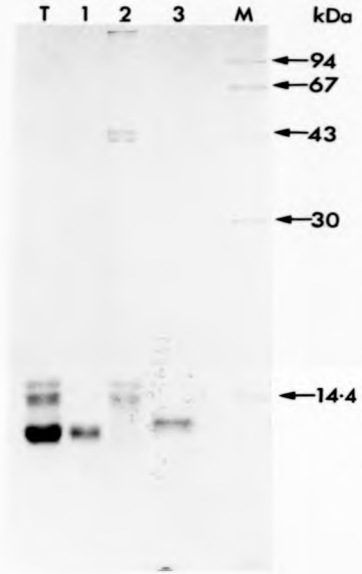

T
1
2
3
M
kDa
94
67
43
30
14·4

Figure R 3(E) Elution profile of the protein from peak 1 from Figure R 3(D) fractionated on a CM-cellulose column

The protein from Peak 1 of the DEAE-Sephacel column was applied to a column (2.5 x 15 cm) of CM-cellulose and the bound protein eluted with a 0-0.15 M NaCl gradient as described in Section M 13. The arrows in Figure R 3(E) indicate the beginning and end of the sodium chloride gradient. Protein still bound to the column was eluted with 0.5 M NaCl in 10 mM Tris-HCL, pH 7.0. Two ml fractions of the bound and unbound protein were collected.

The aborbance at 280 nm of aliquots of the even numbered fractions was measured and plotted against fraction number. The conductivity of every tenth fraction was measured and the corresponding sodium chloride concentration plotted against fraction number.

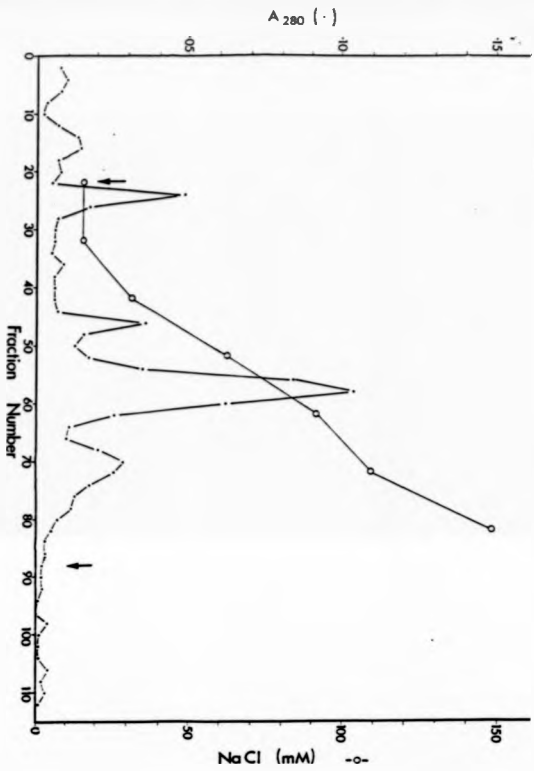

A 280 (·)
.05
.10
.15
0
10
20
30
40
50
60
70
80
90
100
110
Fraction Number
0
50
100
150
NaCl (mM) -o-

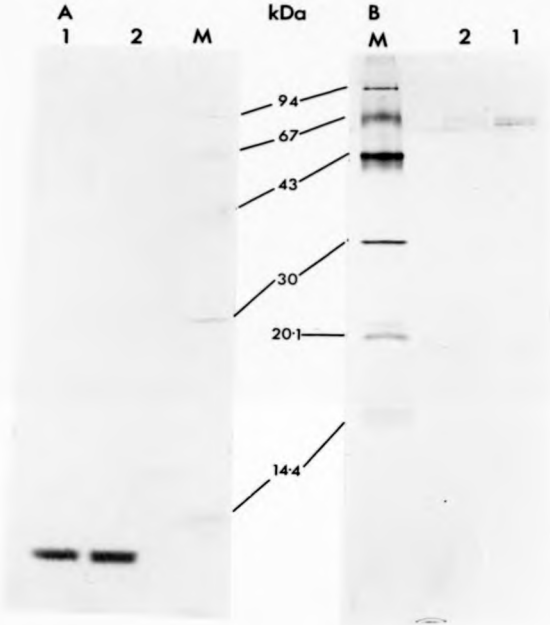
Figure R 3(F) SDS-PAGE analysis of the $A_{280}$ peak shown in Figure R 3(E)

The protein in the major absorbance peak in Figure R 3(E), Li's protein, was precipitated, collected and dialysed as described in Section M 13. Two μg were analysed by SDS-PAGE using a resolving gel of 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide and compared with two μg of the protein before chromatography on CM-cellulose. The proteins were visualised by staining with Coomassie blue (Sub-section M 4(A)): Figure R 3(F), Part A

| | |
|---|---|
| Track 1 | Li's protein before CM-cellulose chromatography, unreduced |
| Track 2 | Li's protein after CM-cellulose chromatography, unreduced |
| Track M | molecular weight markers, reduced |

Duplicate samples were resolved on an identical SDS-polyacrylamide gel and silver stained (Figure R 3(F), Part B).

The molecular weight markers were those described in Section M 2 for use with SDS-PAGE.


A
1
2
M
kDa
B
M
2
1
94
67
43
30
20·1
14·4

Purification of Li's Protein.

The protocol developed for the purification of the major IgE-binding 2S albumin, Li's protein, was based on that of Li et al., [1977]. The original protocol was not practicable mainly because of the large columns required (2.5 x 90 cm, in one case).

The starting material for the purification of Li's protein was the 2S albumin fraction shown in Figure R 2(B), track 2. This fraction was substantially free from proteins of molecular mass greater than about 20 kDa. The basis of Li's protocol, two sequential ion-exchange columns, was retained but using smaller columns [2.5 x 15 cm, in each case]. Desalting was effected by dialysis rather than gel filtration simply for convenience.

Almost complete purification of Li's protein was achieved using the DEAE Sephacel anion-exchanger. Li's protein passed through the column [Figure R 3(C), peak 1 and Figure R 3(D), track 1] whilst the other 2S albumins bound and were partially fractionated by the salt gradient [Figure R 3(C), peaks 2 and 3; Figure R 3(D), tracks 2 and 3]. In particular, a minor component of a molecular weight slightly greater than that of Li's protein was almost completely purified [Figure R 3(D), track 3].

Li's protein was purified to homogeneity by cation-exchange chromatography on CM-cellulose [Figure R 3(E)]. Traces of a 64 kDa doublet, probably lectin, were almost completely removed [compare tracks 1 and 2 of Figure R 3(F), Part B]. Li's protein is not visible in either track 1 or track 2 since it consistently failed to stain with the silver staining protocol employed [Wray et al., 1981].

Approximately one mg of Li's protein was obtained per gramme of castor beans. The yield of Li's protein at each stage could not be determined since there was not a specific assay for the protein.

## Observations on the Chemical Basis of Silver Staining.

Silver staining is in the order of one hundred times more sensitive than Coomassie staining [Switzer et al., 1979 and Oakley et al., 1980]. Many protocols have been developed in an attempt to simplify the procedure and increase the sensitivity [Switzer et al., 1979; Oakley et al., 1980; Merril et al., 1981; Wray et al., 1981; Morrissey, 1981 and Merril et al., 1982]. Despite its evident importance the chemical processes basic to silver staining are poorly understood.

Reduction of silver cations to metallic silver is clearly fundamental to any silver staining protocol. Guevara et al., [1982] developed a procedure similar to that of Wray [1981] used here and proposed a mechanism applicable to protocols of this type involving the reduction of ammoniacal silver nitrate with formaldehyde. It is proposed that ammoniacal silver ions associate with negatively charged groups within protein bands and are subsequently reduced via the oxidation of formaldehyde. Since the gel is incubated in alkaline conditions the principal negatively charged groups will be carboxyl groups. In this model the ability to detect a protein by silver staining depends on the number and, probably, accessibility of groups bearing a negative charge. No account is taken of the ability of the sulphydryl group of cysteine to act as a reducing agent in its own right.

In contrast Chuba and Palchaudhury [1986] emphasized the importance of cysteine residues, particularly in colour silver staining reactions. They demonstrated that outer membrane proteins of E. coli which contain no cysteine failed to silver stain. They also cited several precedents for this behaviour including histone H1 [Nielsen and Brown, 1984],

calmodulin lack cysteine and troponin C contains only a single cysteine residue.

Although the sequence of Li's protein shows a high percentage of cysteine [8.4%] data presented in Section 6 of the Results and Discussion Chapter suggest that these residues may be involved in extensive disulphide bonding. In this case prior reduction of the protein should improve its silver staining properties, a possibility which was not tested. At least one protocol has been developed in which the gel is initially soaked in a reducing agent [Morrissey, 1981].

## Conclusions

It is concluded, on the basis of its molecular weight and band pattern on an SDS-polyacrylamide gel, that the 2S albumin isolated by Li et al., [1977] is the major IgE-binding protein of the unreduced 2S storage albumin fraction. This conclusion is supported by the fact that a protocol based on that of Li et al., [1977] was successfully used to purify the major 2S albumin IgE-binding protein.

Li's protein may be encoded by a multigene family.

SECTION 4

# The Structure and Associating Ability of Li's Protein.

Summary of Results

SDS-PAGE analysis of a sample of reduced Li's protein showed only the large subunit. The small subunit only became visible on the application of a large mass of protein [10 μg].

On a Western blot only the large subunit appeared to bind IgE from the CE pool and was less antigenic than unreduced Li's protein.

On a non-denaturing gel Li's protein migrated as a single, diffuse band of molecular mass approximately 270 kDa.

Figure R 4(A) SDS-PAGE analysis of purified Li's protein

Two μg of Li's protein were reduced by addition of one μl of 0.5 M dithiothreitol (Section M 2) and boiled for three min. The reduced protein was analysed by SDS-PAGE using a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel (Section M 2) and compared with two μg of unreduced Li's protein:

| | |
|---|---|
| Track M | molecular weight markers |
| Track 1 | unreduced Li's protein |
| Track 2 | reduced Li's protein |

The proteins were stained with Coomassie blue (Sub-section M 4(A)). The molecular weight markers were those described in Section M 2 for use in SDS-PAGE.

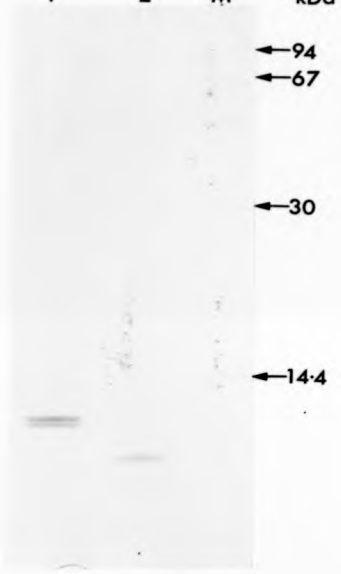
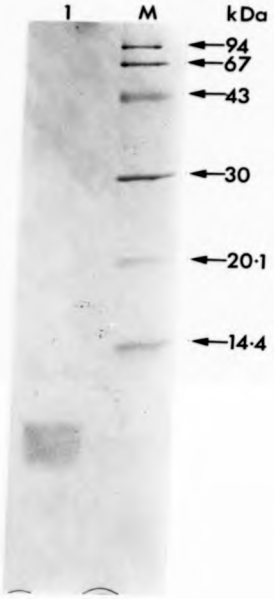

1
2
M
kDa
94
67
30
14·4

Figure R 4(B) SDS-PAGE analysis of 12μg of reduced Li's protein

12 μg of Li's protein was reduced and analysed by SDS-PAGE using a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel as described in Section M 2.

| | |
|---|---|
| Track M | molecular weight markers |
| Track 1 | reduced Li's protein |

The proteins were visualised by Coomassie staining (Sub-section M 4(A)). The molecular weight markers were those described in Section M 2 for use in SDS-PAGE.


1
M
kDa
94
67
43
30
20·1
14·4

## The Subunit Structure of Li's Protein.

Reduction of purified Li's protein yielded a single band of molecular mass about 10 kDa [Figure R 4(A), track 2]. According to Sharief and Li (1982) and as shown in the previous section, Li's protein is composed of two, non-identical subunits of molecular masses 7 kDa and 4 kDa. That only one subunit is seen could be explained by the observation in the previous section that the small subunit either binds Coomassie blue inefficiently or is leached from the gel. In both cases applying a greater amount of protein should help to visualise the small subunit.

In Figure R 4(B) a six times greater mass of Li's protein than that used in Figure R 4(A) appears as a smear in the molecular mass range 8 kDa to 10 kDa. Since the smear extends downwards from the 10 kDa band previously seen in Figure R 4(A), track 2 it is probably the small subunit. There is no indication of the complex large and small subunit band pattern seen in Figure R 3(B).

The molecular mass range is higher than that predicted from the sequence which gives accurate molecular mass estimates of 7 kDa and 4 kDa for the large and small subunits, respectively. This discrepancy is probably due to the anomalous behaviour of small proteins using SDS-PAGE. It was also necessary to extrapolate the calibration curve below the range of the protein standards in order to estimate the molecular mass of Li's protein and its subunit.

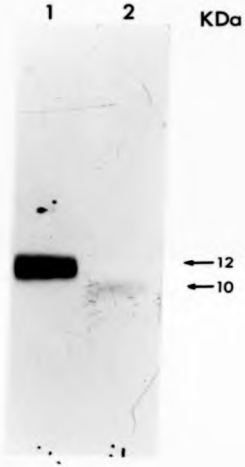
There are at least two other examples of low molecular weight seed storage proteins which are composed of subunits; the napins of *Brassica napus*, rapeseed, [Lonnerdal and Janson, 1972] and a protein from the seeds of *Pisum sativum*, pea, [Gatehouse *et al*., 1985].

Figure R 4(C) Western blot analysis of Li's protein probed with the CB pool

Duplicate two µg samples of Li's protein were resolved by SDS-PAGE using a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel (Section M 2) and blotted onto nitrocellulose (Section M 5). One sample was unreduced and one sample was reduced (Section M 2).

The blotted proteins were probed with the CB pool and the filter was then probed with iodinated anti-IgE (Section M 8) and exposed to film for eight days (Section M 23).

| | |
|---|---|
| Track 1 | unreduced Li's protein |
| Track 2 | reduced Li's protein |


1
2
KDa
12
10

D 4(C)

The IgE-Binding Capacity of Unreduced and Reduced Li's Protein.

Unreduced and reduced samples of Li's protein were blotted and probed with the CB pool [Figure R 4(C)]. Both samples bound IgE but unreduced Li's protein was much more reactive than the reduced form. This observation suggests that three-dimensional conformation is important to the antigenicity and, hence, allergenicity of Li's protein. The disparity between the IgE-binding capacity of reduced and unreduced 2S albumins was also evident in Figure R 2(C), Part B.

Apparently only a single band of 10 kDa binds IgE on the blot of reduced Li's protein. This observation suggests that only the large subunit of Li's protein elicits an IgE response. As discussed earlier in this section there is some evidence to suggest that the small subunit may be leached from an SDS-polyacrylamide gel during staining. The Western blotting protocol used here involves soaking the gel for half an hour in transfer buffer prior to blotting. Leaching of the small subunit at this stage would result in a disproportionately low amount of the small subunit on the filter and could account for its apparent inability to bind IgE.

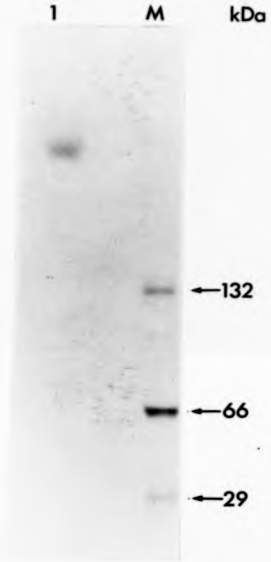
It is important to emphasize that statements describing the IgE-binding capacity of unreduced and reduced Li's protein are based on qualitative judgements. They require to be substantiated by quantitative measurement of the amount of specific IgE bound by a known mass of protein.

Figure R 4(D) Analysis of Li's protein on a non-denaturing gel

Li's protein (10 μg) was analysed on a 4-30% (w/v) acrylamide, non-denaturing gel as described in Section M 3.

| | |
|---|---|
| Track 1 | Li's protein |
| Track M | molecular weight markers |

The proteins were stained with Coomassie blue (Sub-section M 4(A)). The molecular weight markers for non-denaturing gels were those described in Section M 3.


1
M
kDa
132
66
29

Behaviour of Li's Protein on a Non-Denaturing Gel

Under non-denaturing conditions Li's protein migrates as a diffuse band of molecular mass approximately 270 kDa [Figure R 4(D)]. This band may represent a multimer of about 24 monomers, where a monomer is composed of one large and one small subunit. This result was reproducible.

Estimating molecular weight on the basis of mobility on a non-denaturing gel may result in considerable inaccuracy. The approach is limited by the fact that mobility under non-denaturing conditions depends on factors other than molecular weight, including charge and associative ability. A net positive charge, for example, would retard the migration of the protein towards the anode during electrophoresis resulting in a considerable overestimate of molecular weight. Although this is an important qualification it is still possible that Li's protein is capable of associating to form a large multimeric aggregate. This property could be important to the allergenicity of Li's protein.

Allergens are typically of low molecular weight which, at least in the case of inhalant allergens, is a physical constraint imposed if the allergen is to cross the mucosal membranes of the respiratory passages [Marsh, 1975]. It is assumed, then, that Li's protein is denatured when it encounters the nasal membranes so that its effective size is only 11 kDa. A precedent for this proposal is found in the case of the potent antigen, keyhole limpet haemocyanin [KLH]. KLH has an estimated molecular mass, under non-denaturing conditions of $10^3$ kDa [Barte and Campbell, 1959]. Intranasal immunisation of normal or atopic subjects with KLH led to the production of IgE [Salvaggio et al., 1969]. Haemocyanins are composed of associating subunits of molecular mass

about 30 kDa [Pickett et al., 1966]. It is probable, therefore, that the dissociated subunits are absorbed [see also King, 1976].

If Li's protein monomers can reassociate under physiological conditions then pre-B cells of the appropriate antigenic specificity would be presented with a large antigen of highly repetitive structure. This may be an important feature in clonal selection and amplification [Marsh, 1975 and Stanworth, 1973]. Stanworth [1973] further proposed that aggregation may be a general feature of allergens of unusually small molecular weight. In contradiction of this proposal many low molecular weight allergens, such as Ra 5 and Ra 3 of ragweed, show no tendency to aggregate [Marsh, 1975].

## Conclusions

The large subunit of Li's protein is the 10 kDa putative allergen. The small subunit may make no contribution to the allergenicity of Li's protein.

The antigenicity and, hence, allergenicity of Li's protein may be dependant on the three-dimensional conformation of the protein.

Under non-denaturing conditions Li's protein apparently forms a large multimeric aggregate which may be important in eliciting an IgE response.

SECTION 5

# The Ability of Li's Protein to Inhibit Serine Proteases.

## Summary of Results

Li's protein was shown to specifically inhibit trypsin but not chymotrypsin. 0.8 moles of Li's protein were required to fully inhibit one mole of trypsin, consistent with the predicted value of one mole.

Figure R 5(A) Plot of absorbance at 253 nm versus time for the inhibition of trypsin by Li's protein

The trypsin inhibitory activity of Li's protein was determined using the assay described in Section M 15. The absorbance at 253 nm was measured at 20 sec intervals and plotted against time. The masses of Li's protein present in each of the reaction mixtures were:

| | μg |
|---|---|
| $T_o$ | 0 |
| $T_1$ | 0.33 |
| $T_2$ | 0.66 |
| $T_3$ | 0.99 |
| $T_4$ | 1.34 |
| $T_5$ | 1.65 |

The standard mass of trypsin present in each reaction mixture was 1.65 μg.

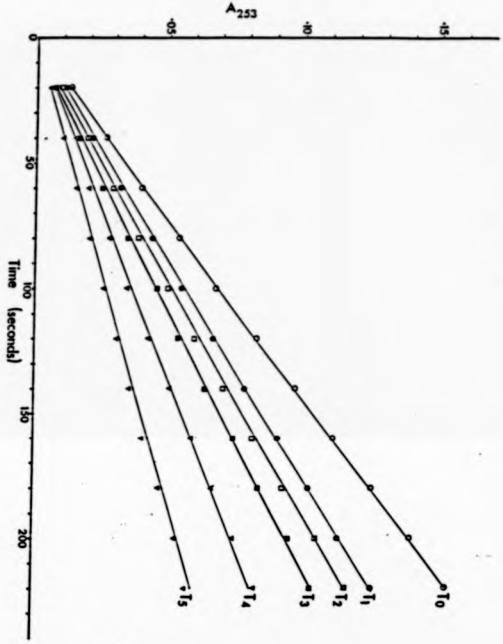

$A_{253}$
·05
·10
·15
0
50
100
150
200
Time (seconds)
$T_0$
$T_1$
$T_2$
$T_3$
$T_4$
$T_5$

Figure R 5(B) Plot of percentage activity versus μg inhibitor for trypsin in the presence of a range of Li's protein concentrations

The gradients of the plots of $A_{253}$ versus time (Figure R 5(B)) were taken as a measure of trypsin activity in the presence of five different concentrations of Li's protein, $T_o$-$T_5$. The value of each gradient was expressed as a percentage of the gradient value for the plot of $A_{253}$ versus time in the absence of Li's protein, $T_o$. The percentage activities were plotted against the number of μg of Li's protein in each reaction mixture and the plot extrapolated until it intercepted the x-axis, giving a value for the number of μg of inhibitor required to completely inhibit the standard mass of trypsin in each reaction (1.65 μg).

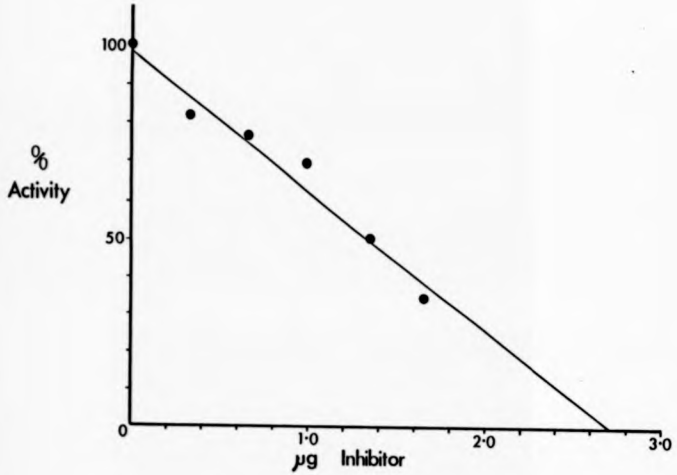

100
%
Activity
50
0
1·0
2·0
3·0
µg Inhibitor

Figure R 5(C) Plot of absorbance at 256 nm versus time for the inhibition of chymotrypsin by Li's protein

The chymotrypsin inhibitory activity of Li's protein was determined using the assay described in Section M 15 except that only two reaction mixtures were assayed:

| | μg |
|---|---|
| $T_o$ | 0 |
| $T_1$ | 10 |

the mass of chymotrypsin in each case was 10 μg.

The absorbance at 256 nm was measured at 20 sec intervals and plotted against time.

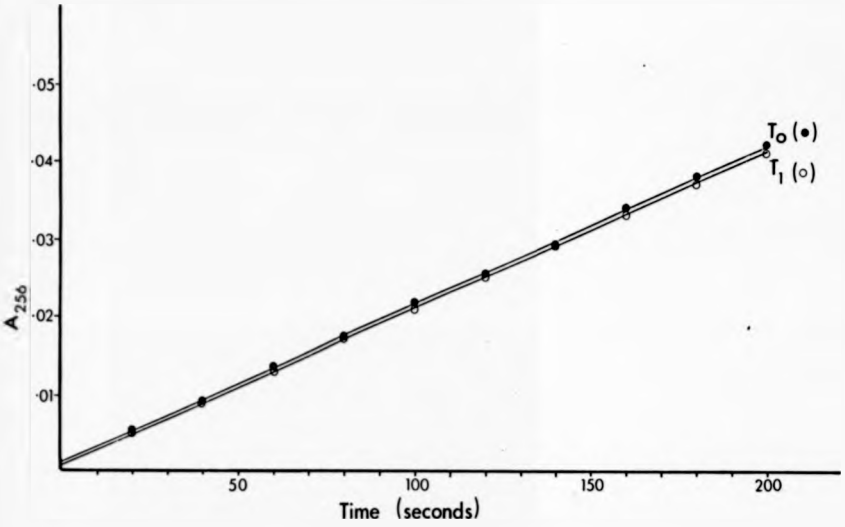

·05
·04
·03
·02
·01
$A_{256}$
50
100
150
200
Time (seconds)
$T_0$ (•)
$T_1$ (○)

Figure R 5(D) Plot of absorbance at 253 nm versus time for the inhibition of trypsin by lima bean trypsin inhibitor

The trypsin inhibitory activity of lima bean trypsin inhibitor was determined using the assay described in Section M 15. The absorbance at 253 nm was measured at 20 sec intervals and plotted against time. The masses of lima bean trypsin inhibitor present in each of the reaction mixtures were:

| | μg |
|---|---|
| $T_o$ | 0 |
| $T_1$ | 0.33 |
| $T_2$ | 0.66 |
| $T_3$ | 0.99 |
| $T_4$ | 1.34 |
| $T_5$ | 1.65 |

The standard mass of trypsin present in each reaction mixture was 1.65 μg.

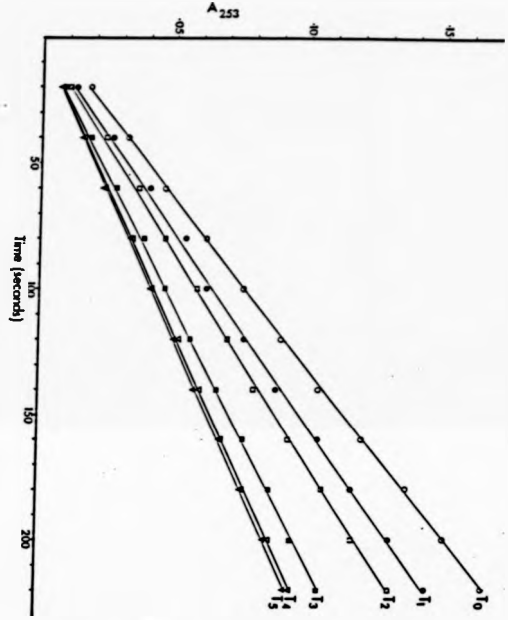

$A_{253}$
.05
.10
.15
50
100
150
200
Time (seconds)
$T_0$
$T_1$
$T_2$
$T_3$
$T_4$
$T_5$

Figure R 5(E) Plot of absorbance at 256 nm versus time for the inhibition of chymotrypsin by lima bean trypsin inhibitor

The chymotrypsin inhibitory activity of lima bean trypsin inhibitor was determined using the assay described in Section M 15. The absorbance at 256 nm was measured at 20 sec intervals and plotted against time (sec). The masses of lima bean trypsin inhibitor in each reaction mixture were:

| | μg |
|---|---|
| $T_0$ | 0 |
| $T_1$ | 5 |
| $T_2$ | 10 |
| $T_3$ | 15 |
| $T_4$ | 20 |
| $T_5$ | 25 |

The standard mass of chymotrypsin in each mixture was 10 μg.

$A_{256}$
·01
·02
·03
·04
50
100
150
200
Time (seconds)
$T_0$
$T_1$
$T_2$
$T_3$
$T_4$
$T_5$

Figure R 5(F) Plot of percentage activity versus μg inhibitor for trypsin in the presence of a range of lima bean trypsin inhibitor concentrations

The gradients of the plots of $A_{253}$ versus time (Figure R 5(D)) were taken as a measure of trypsin activity in the presence of five different concentrations of lima bean trypsin inhibitor, $T_0$-$T_5$. The value of each gradient was expressed as a percentage of the gradient value for the plot of $A_{253}$ versus time in the absence of lima bean trypsin inhibitor, $T_0$.

The percentage activities were plotted against the number of μg of lima bean trypsin inhibitor in each reaction mixture and the plot extrapolated until it intercepted the x-axis, giving a value for the number of μg of inhibitor required to completely inhibit the standard mass of trypsin in each reaction (1.65 μg).

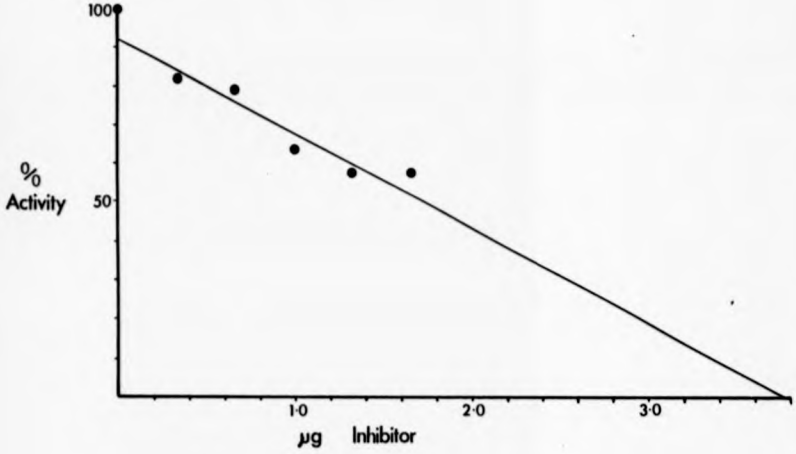
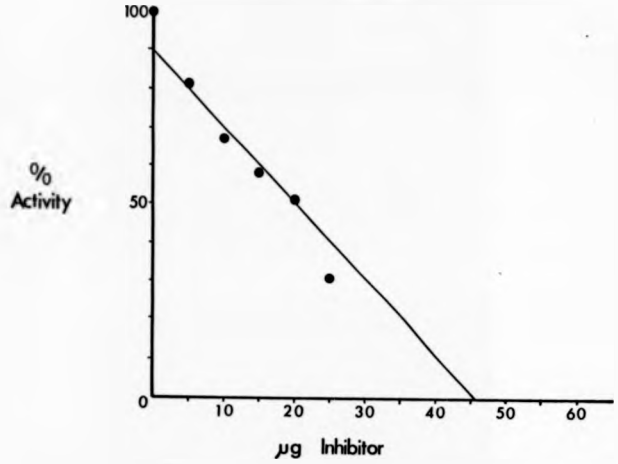
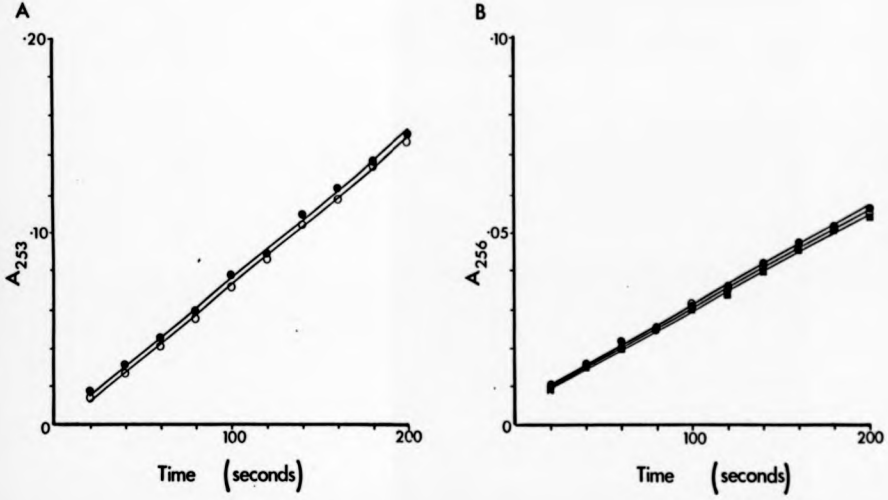

100
%
Activity
50
1·0
2·0
3·0
µg Inhibitor

Figure R 5(G) Plot of percentage activity versus μg inhibitor for chymotrypsin in the presence of a range of lima bean trypsin inhibitor concentrations

The gradients of the plots of $A_{256}$ versus time (Figure R 5(E)) were taken as a measure of chymotrypsin activity in the presence of five different concentrations of lima bean trypsin inhibitor, $T_0$-$T_5$. The value of each gradient was expressed as a percentage of the gradient value for the plot of $A_{256}$ versus time in the absence of lima bean trypsin inhibitor, $T_0$.

The percentage activities were plotted against the number of μg of lima bean trypsin inhibitor in each reaction mixture and the plot extrapolated until it intercepted the x-axis, giving a value for the number of μg of inhibitor required to completely inhibit the standard mass of chymotypsin in each reaction (10 μg).


100
50
0
% Activity
10
20
30
40
50
60
µg Inhibitor


A
·20
·10
0
$A_{253}$
100
200
Time (seconds)
B
·10
·05
0
$A_{256}$
100
200
Time (seconds)

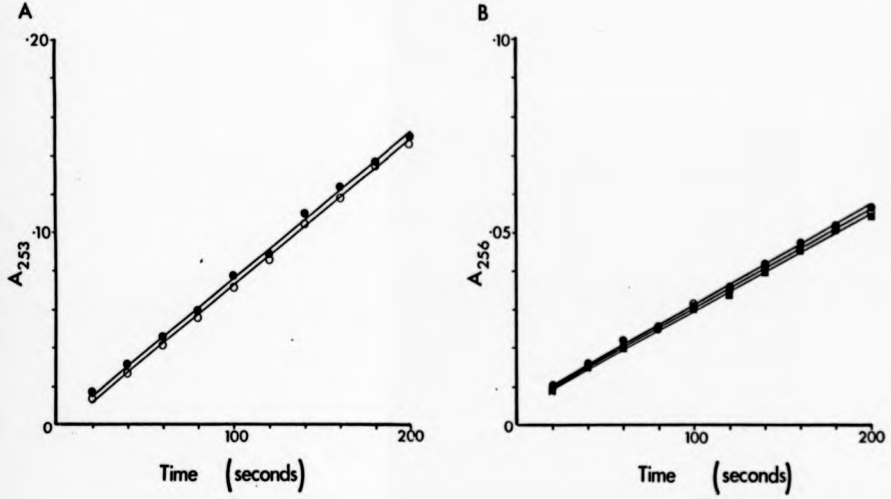

A
·20
·10
0
$A_{253}$
100
200
Time (seconds)
B
·10
·05
0
$A_{256}$
100
200
Time (seconds)

Figure R 5(H) The ability of bovine serum albumin to inhibit trypsin and chymotrypsin and of Li's protein to inhibit chymotrypsin

The trypsin inhibitory activity (Figure R 5(H), Part A) and the chymotrypsin inhibitory activity (Figure R 5(H), Part B) of bovine serum albumin (BSA) was determined using the assay described in Section M 15. The absorbance at 253 nm, in the case of trypsin, and at 256 nm, in the case of chymotrypsin, was measured at 20 sec intervals and plotted against time (sec). Only two reaction mixtures were used in each case:

Part A

| | mass of BSA ($\mu$g) |
|---|---|
| $T_o$ | 0 |
| $T_b$ | 25 |

The standard mass of trypsin in each reaction mixture was 1.65 $\mu$g.

Part B

| | mass of BSA ($\mu$g) |
|---|---|
| $T_o$ | 0 |
| $T_b$ | 25 |

The standard mass of chymotrypsin in each reaction mixture was 10 $\mu$g.

In addition the chymotrypsin inhibitory activity of Li's protein was also determined. A single reaction mixture containing 10 μg chymotrypsin and 25 μg Li's protein was used.

Figure R 5(H), Part A:

| | |
|---|---|
| Solid circles | Trypsin activity in the absence of BSA. |
| Open circles | Trypsin activity in the presence of BSA. |

Figure R 5(H), Part B:

| | |
|---|---|
| Solid circles | Chymotypsin activity in the absence of BSA or Li's protein |
| Open circles | Chymotrypsin activity in the presence of Li's protein |
| Solid squares | Chymotrypsin activity in the presence of BSA. |

## Inhibition of Trypsin by Li's Protein.

Sharief and Li [1982] demonstrated sequence homology between the large subunit of Li's protein and the Bowman-Birk serine protease inhibitor of lima bean. The homologous region included the reactive site residues for trypsin inhibition, suggesting that Li's protein may also be a serine protease inhibitor specific for trypsin. To investigate this possibility the ability of Li's protein to inhibit the hydrolysis of synthetic substrates of trypsin and chymotrypsin was measured spectrophotometrically.

A range of reaction mixes were set up in which the concentration of protease was constant but that of the inhibitor was varied. For each reaction mix absorbance was plotted against time and the gradient of the plot used as a measure of enzyme activity. The value for each gradient was expressed as a percentage of enzyme activity in the absence of inhibitor. The percentage activities were then plotted against the number of microgrammes [$\mu$g] of inhibitor present in each reaction mix. Extrapolation of this plot to the point at which it intercepted the x-axis gave an estimate of the amount of inhibitor required to fully inhibit the standard mass of enzyme in each reaction mix.

The graphs of absorbance vs. time and percentage activity vs. $\mu$g. inhibitor for trypsin in the presence of Li's protein are shown in Figures R 5(A) and R 5(B), respectively. From Figure R 5(B), one $\mu$g of Li's protein will fully inhibit 1.65 $\mu$g trypsin; expressed in moles, 0.8 moles of Li's protein are required to fully inhibit one mole of trypsin.

The ability of Li's protein to inhibit chymotrypsin was also investigated. Absorbance vs. time was plotted for chymotrypsin in the absence of Li's protein and also for a single reaction mixture with

chymotrypsin in the presence of Li's protein. The mass of Li's protein used was fifteen times that used in the trypsin inhibition assay. Li's protein had no appreciable inhibitory effect on chymotrypsin [Figure R 5(C)]. This conclusion is supported by the absence of a potential chymotrypsin inhibition site in the sequence of Li's protein. Sequence comparisons between a number of serine protease inhibitors have shown that the amino acid pairs at the site of chymotrypsin inhibition are tryptophan-serine, phenylalanine-serine, tyrosine-serine or leucine-serine, none of which are found in the sequence of Li's protein [Figure I 1].

As a control the trypsin and chymotrypsin inhibitory abilities of the lima bean serine protease inhibitor [LTI] were assayed. LTI is a double-headed inhibitor in that it has two reactive sites, one specific for trypsin and one specific for chymotrypsin [Tan and Stevens, 1971]. Figures R 5(D) and R 5(E) show the plots of absorbance vs. time for, respectively, trypsin and chymotrypsin both in the presence of LTI. Figures R 5(F) and R 5(G) show the corresponding plots of percentage activity vs. μg. inhibitor. From these data 0.9 moles of LTI are required to inhibit 1 mole of trypsin and 1.8 moles of LTI are required to inhibit 1 mole of chymotrypsin.

A further control was to assay the inhibitory activity of bovine serum albumin on trypsin and chymotrypsin. The object of this control was to demonstrate that Li's protein and LTI were not exercising a non-specific inhibitory activity on the proteases tested. Figure R 5(H), shows that bovine serum albumin had no appreciable inhibitory effect on trypsin [Figure R 5(H), Part A] or chymotrypsin [Figure R 5(H), Part B]. The reaction mechanism of serine protease inhibition by protein inhibitors has been extensively studied (for a review see Laskowski and Kato, 1980). Inhibition is competitive, the reaction site of the inhibitor interacts with that of the protease and forms a stable,

equimolar complex. The sequence of Li's protein (Figure I 1) suggests that the protein has a single inhibitory site specific for trypsin. One mole of Li's protein would be expected to inhibit one mole of trypsin. The same argument applies to LTI which has a single trypsin inhibitory site and a single chymotrypsin inhibitory site (Tan and Stevens, 1971). The observed values of 0.8 moles of Li's protein and 0.9 moles of LTI required to fully inhibit one mole of trypsin are consistent with this model.

The observed value of 1.8 moles of LTI required to fully inhibit one mole of chymotrypsin is almost twice the predicted value, an observation which was reproducible. A trivial explanation for this observation is that the assay conditions, as specified in the Worthington biochemicals catalogue, were not suited for use with the preparation of chymotrypsin supplied by Sigma.

An unusual feature of Li's protein is its subunit composition. 2S seed storage albumins have been identified which are composed of subunits linked by disulphide bonds (see Sub-section I 4(D)). In relation to this class of storage proteins the subunit structure of Li's protein is not unusual. In contrast, a literature search by the author failed to find an example of a plant serine protease inhibitor composed of covalently-linked subunits.

Table R 1 The Inhibitory Activities of Lima Bean Trypsin Inhibitor and Li's Protein

The inhibitory activities of lima bean trypsin inhibitor and Li's protein against trypsin and chymotrypsin were determined as described in Sub-section D 5(B). Table R 1 presents the predicted and observed values for the number of moles of each inhibitor required to inhibit one mole of each protease.

| | Trypsin | Chymotrypsin | |
|---|---|---|---|
| LTI | 1.0 | 1.0 | Predicted Value |
| | 0.9 | 1.8 | Observed Value |
| Li's protein | 1.0 | Ineffective | Predicted Value |
| | 0.8 | Ineffective | Observed Value |

The Significance of the Trypsin Inhibitory Ability of Li's Protein.

That Li's protein is a trypsin inhibitor has been clearly demonstrated. This property may be important to the allergenicity of Li's protein. It is well established that destruction of the three-dimensional configuration of a protein allergen usually destroys its allergenic properties, probably due to the loss of immunodominant conformational determinants [Marsh, 1975]. The ability of Li's protein to resist digestion by trypsin, at least, may prolong its life-span under physiological conditions. A precedent for this idea is found in the experiments of Chang and Marsh [1974] in which ragweed allergen AgE, resistant to proteolysis by trypsin and chymotrypsin, was shown to be more antigenic and allergenic than the readily degradable rye Group 1 allergen.

The trypsin inhibitory ability of Li's protein also supports the earlier conclusion [Results and Discussion, Section 3] that the major IgE-binding 2S albumin studied here, Li's protein, is the same protein as that purified by Li et al. [1977]. The sequence of the protein purified by Li predicted that it is a trypsin inhibitor but not a chymotrypsin inhibitor.

## Conclusions

Li's protein specifically inhibits trypsin, a property which may increase its life-span under physiological conditions and, thereby, enhance its ability to elicit an IgE response.

The trypsin inhibitory ability of Li's protein is consistent with the earlier conclusion [Results and Discussion, Section 3], that the major IgE-binding 2S albumin studied here, Li's protein, is the same protein as that purified by Li *et al*. [1977].

SECTION 6

# The Specificity of IgG Antibodies Raised Against Unreduced Li's Protein.

Summary of Results

Western blot analysis showed that IgG antibody raised against unreduced Li's protein [anti-Li's protein antibodies] reacted strongly with unreduced Li's protein and only weakly with reduced Li's protein. Acetylation of reduced Li's protein almost completely abolished its reactivity with anti-Li's protein antibodies. The other components of the 2S albumin fraction and the 7S lectins did not react with anti-Li's protein antibodies.

Anti-Li's protein antibodies cross-reacted strongly with two different doublets in the reduced and unreduced crystalloid fractions. The upper and lower bands of the unreduced, cross-reactive doublet had molecular masses of 55.6 kDa and 53.7 kDa respectively, while the upper and lower bands of the reduced, cross-reactive doublet had molecular masses of 34.7 kDa and 33.7 kDa, respectively. The reduced, cross-reactive protein was shown to be composed of at least one subunit of the unreduced, cross-reactive protein.

Western blot analysis showed that anti-Li's protein antibodies, raised against Li's protein purified during this study, reacted strongly with a sample of the castor bean 2S albumin purified by Li *et al*. [1977].

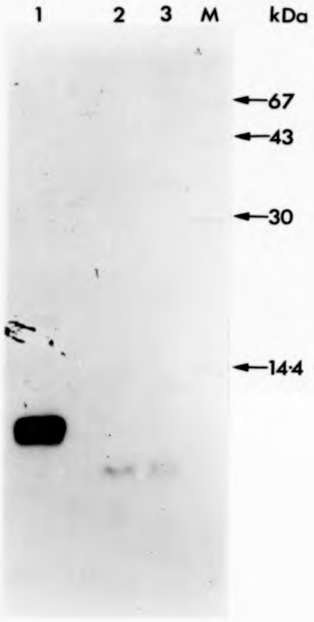
Figure R 6(A) Western blot analysis of Li's protein probed with IgG antibodies raised against native Li's protein

Three aliquots containing two $\mu$g each of Li's protein were resolved by SDS-PAGE using a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel (Section M 2) and blotted onto nitrocellulose (Section M 5). The protein in one aliquot was unreduced, one sample was reduced and one sample was reduced and acetylated (Section M 2).

The blotted proteins were probed with rabbit IgG antibodies raised against native Li's protein and the filter was then probed with iodinated protein A (Section M 7) and exposed to film for three days (Section M 23).

| | |
|---|---|
| Track 1 | unreduced Li's protein |
| Track 2 | reduced Li's protein |
| Track 3 | reduced and carboxymethylated Li's protein |
| Track M | molecular weight markers |

Radiolabelled molecular weight markers for use in SDS-PAGE were those described in Section M 2.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kDa
67
43
30
14·4

The Antigenicity of Li's Protein is Dependant on Three-Dimensional Conformation.

Unreduced Li's protein reacts strongly with IgG antibodies raised against it, anti-Li's protein antibodies [Figure 6(A), track 1]. Li's protein treated with the reducing agent dithiothreitol reacts weakly with anti-Li's protein antibodies [Figure R 6(A), track 2]. Almost complete loss of recognition by anti-Li antibodies is achieved by treating reduced Li's protein with iodoacetamide [Figure R 6(A), track 3].

These results suggest that the antigenic determinants of Li's protein, those features of the protein which elicit an antibody response, are highly dependant on the three-dimensional conformation of the protein. That disulphide bonds are important in stabilising the conformation of Li's protein may be infered not only from the loss of antigenicity on reduction of the protein but also on the basis of the large percentage of cysteine residues in the sequence [8.4%] and the known contribution of disulphide bonds to the three-dimensional conformation of many serine protease inhibitors [Laskowski and Kato, 1980 and see Figure I 3].

Antigenic determinants are typically located on the protein surface and are sensitive to conformational change and amino acid substitutions or modifications [see Sub-section I 3(G)]. In this respect the ability of iodoacetamide to almost completely abolish the recognition of reduced Li's protein by anti-Li's protein antibodies is probably due to the acetylation of cysteine residues.

In structural terms antigenic determinants can be divided into two broad categories; those composed of amino acids sequentially linked to

each other by peptide bonds, termed continuous determinants, and those composed of spatially adjacent residues brought together by the folding of the protein, termed discontinuous determinants; [Atassi et al., 1976; Lee and Atassi, 1976]. Proteins apparently bearing only discontinuous determinants, such as lysozyme, are very sensitive to conformational change [Atassi, 1984]. It is likely, then, that the antigenic determinants of Li's protein are principally discontinuous. The fact that attempts to raise antibodies against reduced Li's protein were unsuccessful supports this idea. It is possible, though, that the antigenic determinants of Li's protein are continuous, located on disulphide-bonded loops and rendered inaccessible on reduction of the protein.

It was noted in Sub-section D 4(C) of this Chapter that the conformational dependence of antigenicity may also be a feature of the IgE response to Li's protein [Figure R 4(C)]. The same structural feature of the protein may be responsible for eliciting both an IgG and an IgE response. It has recently been proposed that the human IgG and IgE responses against allergenic chironomid haemoglobins are both stimulated by the same determinants [Baur, 1986].

Figure R 6(B) The ability of the 2S albumin purified by Li et al (1977) to bind IgG antibodies raised against Li's protein purified during this study

Two μg of Li's protein purified during this study and two μg of the equivalent protein purified by Li et al. (1977), a gift from Dr. S. S. Li, were resolved by SDS-PAGE (Section M 2) using a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel.

The proteins were blotted onto nitrocellulose (Section M 5), probed with IgG antibodies raised against native Li's protein purified during this study (Section M 16) and then with iodinated protein A (Section M 7). The filters were exposed to film for 48 h (Section M 23).

| | |
|---|---|
| Track 1 | 2S albumin purified by Li et al. (1977), unreduced |
| Track 2 | Li's protein purified during this study, unreduced |

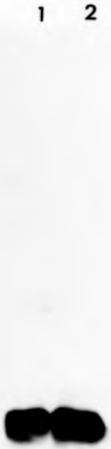
1 2

Figure R 6(C) Specificity of the anti-Li's protein IgG antibodies reactivity with other water-soluble castor bean proteins

Ten μg of total, water-soluble castor bean proteins, isolated as described in Section M 12, and two μg of unreduced Li's protein were resolved by SDS-PAGE on a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel (Section M 2) and blotted onto nitrocellulose (Section M 5).

The filter was probed with anti-Li's protein antibodies then with iodinated protein A (Section M 7) and exposed to film for three days (Section M 23).

| | |
|---|---|
| Track 1 | total, water-soluble castor bean protein, unreduced |
| Track 2 | Li's protein, unreduced |

The molecular weight markers were resolved on the same gel but blotted onto a separate nitocellulose filter and their positions recorded in pencil. Immediately prior to exposure of the filter to film $^{35}$S-methionine-labelled ink was spotted onto the pencil marks. The molecular weight markers were those described in Section M 2 for use in SDS-PAGE.


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20·1
14·4

Figure R 6(D) Specificity of the anti-Li's protein IgG antibodies: reactivity with the crystalloids

Duplicate samples of 10 µl of castor bean crystalloids isolated as described in Section M 12A were resolved by SDS-PAGE on a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel (Section M 2) and blotted onto a nitrocellulose filter (Section M 5). One sample was reduced and one sample was unreduced (Section M 2).

The filter was probed with anti-Li's protein IgG antibodies then with iodinated protein A (Section M 7) and exposed to film for four days. The molecular weight markers and the determination of their positions are as described in the legend to Figure R 6(C).

| | |
|---|---|
| Track 1 | unreduced crystalloid protein |
| Track 2 | reduced crystalloid protein |

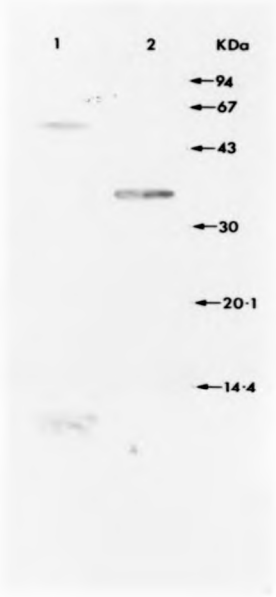
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Figure R 6(E) SDS-PAGE analysis of reduced and unreduced crystalloid protein

Duplicate 10 µg samples of crystalloid protein isolated as described in Sub-section M 12(A) were resolved by SDS-PAGE using a 10% (w/v) acrylamide, 0.26% (w/v) bisacrylamide resolving gel (Section M 2). The gel was stained with Coomassie blue (Sub-section M 4(A)).

| | |
|---|---|
| Track 1 | unreduced crystalloid protein |
| Track 2 | reduced crystalloid protein |
| Track M | molecular weight markers |

The molecular weight markers are as described in Section M 2. The arrows indicate the cross-reactive doublets (CRDs) of the reduced and unreduced crystalloid fraction.

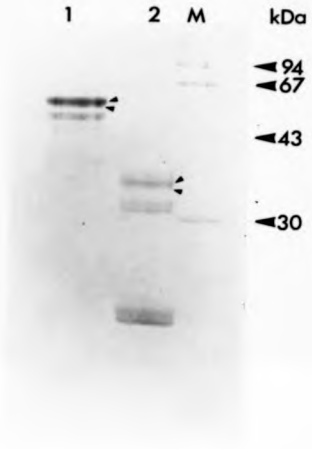
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Figure R 6(F) Identification of the reduced and unreduced cross-reactive doublets

Duplicate 10 μg samples of reduced and unreduced crystalloids were resolved by SDS-PAGE (Section M 2) using a 15% (w/v) acrylamide, 0.26% (w/v) bisacrylamide resolving gel and stained with Coomassie blue (Sub-section M 4(A)). The putative cross-reactive doublets were cut out of the gel with a razor blade, inserted into separate wells of a second, identical SDS-polyacrylamide gel and re-electrophoresed along with duplicate 10 μg samples of reduced and unreduced crystalloids.

The reduced and unreduced proteins were blotted onto separate nitrocellulose filters (Section M 5), probed with anti-Li's protein IgG antibodies and then with iodinated protein A (Section M 7). The filters were exposed to film for four days (Section M 23). The $^{35}S$-labelled molecular weight markers were those described in Section M 2.

Part A

| | |
|---|---|
| Track 1 | total crystalloid protein, unreduced |
| Track 2 | cross-reactive doublet, unreduced |

Part B

| | |
|---|---|
| Track 1 | cross-reactive doublet, reduced |
| Track 2 | total crystalloid protein, reduced |
| Track M | molecular weight markers |

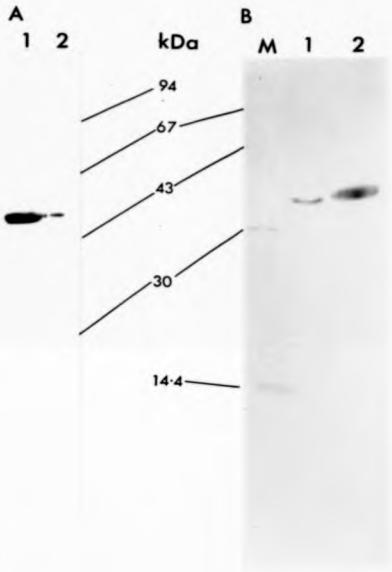
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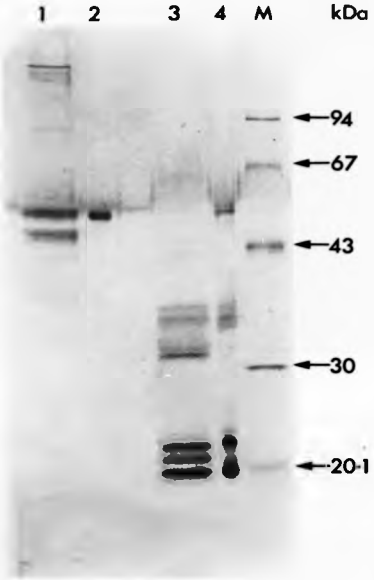
Figure R 6(G) Relationship between the unreduced and reduced cross-reactive crystalloid doublets

Duplicate 10 $\mu$g samples of unreduced crystalloid protein were resolved by SDS-PAGE (Section M 2) and stained with Coomassie blue (Sub-section M 4(A)). The cross-reacting doublet was cut out of each track. One of the gel slices was soaked in 200 $\mu$l of sample buffer, including 10 $\mu$l of 0.5 M DTT, for 10 min at 60$^{o}$C. The reduced gel slice was re-electrophoresed beside a sample (one $\mu$g) of reduced crystalloid protein. The unreduced gel slice was re-electrophoresed next to a sample (one $\mu$g) of unreduced crystalloid protein. The resolved proteins were silver stained (Section M 4(B)). Molecular weight markers were those described in Section M 2 for use in SDS-PAGE. In all cases a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel was used.

| | |
|---|---|
| Track 1 | total crystalloid protein, unreduced |
| Track 2 | cross-reactive doublet, unreduced |
| Track 3 | total crystalloid protein, reduced |
| Track 4 | cross-reactive doublet, reduced |
| Track M | molecular weight markers |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D 6(C)

The Specificity of Anti-Li's Protein Antibodies.

Western blot analysis of the water-soluble storage proteins probed with anti-Li's protein antibodies showed that the antibodies were specific for Li's protein [Figure R 6(C)]. The other proteins of the 2S storage albumin fraction are, therefore, immunologically unrelated to Li's protein. This result contradicts the suggestion in Sub-section D 3(B) of this Chapter that all the proteins of the 2S storage albumin fraction are composed of the same group of subunits.

Western blot analysis of a sample of the 2S albumin isolated by Li et al. (1977) and sequenced by Sharief and Li (1982) showed that this protein reacted strongly with IgG antibodies raised against Li's protein purified during this study [Figure R 6(B), track 1]. This observation strongly supports the earlier conclusion that the protein purified and sequenced by Li and colleagues is the same as the major 2S IgE-binding protein.

Probing reduced and unreduced cystalloid protein with anti-Li's protein antibodies revealed a strong cross-reaction with a doublet in the unreduced crystalloid fraction and a lower molecular weight doublet in the reduced crystalloid fraction [Figure R 6(D)]. The upper and lower bands of the unreduced cross-reactive doublet [unreduced CRD] have molecular masses of 55.6 kDa and 53.7 kDa, respectively, while the upper and lower bands of the reduced cross-reactive doublet [reduced CRD] have molecular masses of 34.7 kDa and 33.7 kDa, respectively.

The crystalloid storage proteins have been extensively characterised [for a detailed discussion see the Introduction, Sub-section I 4(B)]. In brief, they are a heterogeneous complex of at least six oligomers of approximate molecular mass 330 kDa. Each subunit is

composed of an acidic and a basic polypeptide of molecular masses approximately 30 kDa and 20 kDa, respectively, which are linked by disulphide bonds. The molecular weight of the reduced CRD suggests that it is an acidic polypeptide and as such is probably a component of the unreduced CRD. SDS-PAGE analysis of the reduced and unreduced crystalloid proteins is shown in Figure R 6(E) and the cross-reactive doublets indicated with arrows. In view of the complexity of the reduced and unreduced crystalloid fraction the specificity of the cross-reaction is striking.

To confirm that the reduced and unreduced CRDs had been correctly identified the Coomassie-stained doublets were excised from an SDS-polyacrylamide gel, blotted and probed with anti-Li's protein antibodies. Figure R 6(F) shows that both the unreduced CRD, Figure R 6(F), Part A, and the reduced CRD, Figure R 6(F), Part B, had been correctly identified. The reduced CRD appears as a single band because, inadvertantly, only one band of the doublet had been re-electrophoresed and blotted.

To test the suggestion that the reduced CRD is the acidic polypeptide derived from the unreduced CRD the latter was cut out of an SDS-polyacrylamide gel, soaked in dithiothreitol and re-electrophoresed. Silver staining revealed that the unreduced CRD was composed of at least one acidic polypeptide and two basic polypeptides [Figure R 6(G), track 4]. The acidic polypeptide corresponded to at least one band of the reduced CRD [Figure R 6(G), track 3].

The ability of the cross-reactive crystalloid protein to bind anti-Li's protein antibodies is not affected by reduction. This observation is surprising since, as has been discussed in this section, anti-Li's protein antibodies show a marked specificity for unreduced rather than reduced Li's protein. A possible explanation for this discrepancy is that Li's protein and the cross-reactive crystalloid protein share a

common antigenic determinant. It is proposed that this determinant is continuous and is located on a disulphide-bonded loop in Li's protein. Reduction of Li's protein renders the determinant inaccessible to anti-Li's protein antibodies. In the case of the crystalloid protein the common determinant is assumed to be located on a region of the protein not stabilised by disulphide bonds and which would be largely unaffected by reduction. Hence, anti-Li's protein antibodies can bind with equal facility to the reduced and unreduced CRD. Basic to this argument is the assumption that the cross-reaction reflects sequence homology between Li's protein and the crystalloid protein.

## Conclusions

The antigenicity of Li's protein is highly dependent on the three-dimensional conformation of the protein. This observation concurs with similar observations made in Sub-section D 4(B) of this Chapter where reduced and unreduced Li's protein were probed with the CB pool. Together they suggest that the allergenicity of Li's protein is dependent on the three-dimensional conformation of the protein.

Li's protein is immunologically unrelated to the other 2S storage albumins. It is unlikely, then, that all the 2S storage albumin proteins are composed of the same subunits, a suggestion made in Sub-section D 3(B) of this Chapter.

A doublet of two acidic crystalloid polypeptides cross-reacts strongly with anti-Li's protein antibodies. Li's protein and the crystalloid doublet may have sequence homology.

SECTION 7

# Characterisation of the Cross-Reactivity Observed Between Anti-Li's Protein Antibodies and the Reduced CRD.

## SECTION 7

# Characterisation of the Cross-Reactivity Observed Between Anti-Li$^a$ Protein Antibodies and the Reduced CRD.

## Summary of Results

Fragments of the reduced CRD were generated using V8 protease. A single fragment reacted with anti-Li's protein antibodies on a Western blot.

Crystalloid protein treated with N-chlorosuccinimide, which specifically cleaves tryptophanyl peptide bonds, did not react with anti-Li's protein antibodies even though most of the protein was not cleaved.

Western blot analysis of protein extracts from barley and wheat was used to try to explain the crystalloid cross-reactivity in terms of a seed storage protein superfamily of which Li's protein is a member. The results were inconclusive.

The CB pool was shown to react with the same unreduced crystalloid doublet as the anti-Li's protein IgG antibodies. The reduced crystalloid proteins failed to react with the CB pool.

Figure R 7(A) Establishing conditions for the partial proteolysis of the reduced, cross-reactive crystalloid doublet using V8 protease

Four 10 µg samples of reduced crystalloid protein were resolved by SDS-PAGE (Section M 2) and stained with Coomassie blue (Sub-section M 4(A)). The reduced CRD was cut out of each track using a razor blade and each inserted into separate tracks of a second SDS-polyacrylamide gel. 20 µl of three stock concentrations of Straphylococcus aureus V8 protease were layered onto three of the gel slices and digestion allowed to proceed as described in Section M 11. The fourth gel slice was not treated with protease. The resolved digestion products were visualised by silver staining (Sub-section M 4(B)). The molecular weight markers were those described in Section M 2 for use in SDS-PAGE.

| | |
|---|---|
| Track 1 | undigested, reduced CRD |
| Track 2 | reduced CRD digested with 20 µl of V8 protease (0.5 µg/ml). |
| Track 3 | reduced CRD digested with 20 µl of V8 protease (5.0 µg/ml). |
| Track 4 | reduced CRD digested with 20 µl of V8 protease (50.0 µg/ml). |
| Track 5 | 20 µl of V8 protease (50.0 µg/ml) |

In each case a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel was used.

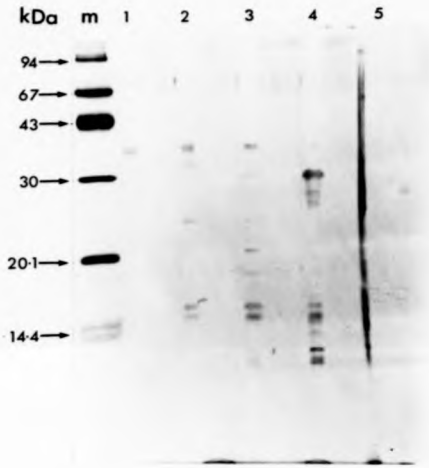
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Figure R 7(B) Identification of a reduced CRD fragment which binds anti-Li's protein IgG antibodies

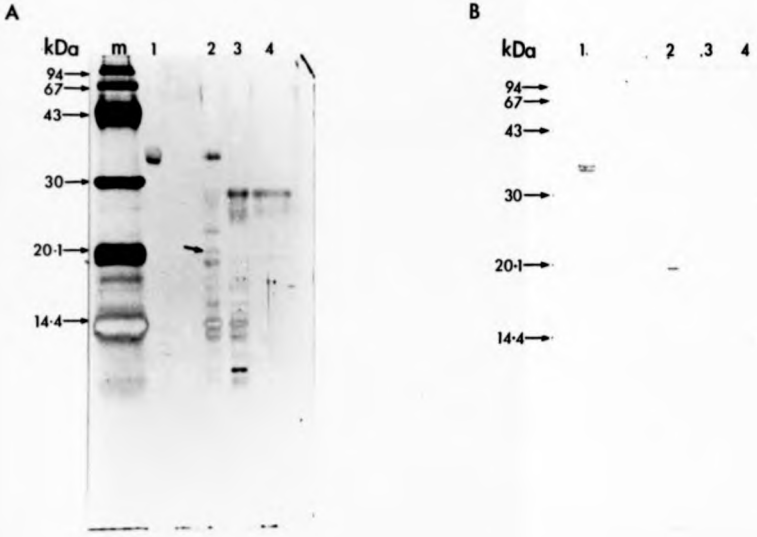
Six samples of the reduced CRD were excised from an SDS-polyacrylamide gel as described in the legend to Figure R 7(A).

The following groups of samples were resolved, in duplicate, on a second SDS-polyacrylamide gel:

| | |
|---|---|
| Track 1 | reduced CRD, undigested |
| Track 2 | reduced CRD, digested with 20 µl of 5.0 µg/mg stock V8 protease. |
| Track 3 | reduced CRD, digested with 20 µl of 50.0 µg/ml stock V8 protease |
| Track 4 | 20 µl, 50 µg/ml stock V8 protease |
| Track M | molecular weight markers |

One group was visualised by silver staining (Sub-section M 4(B)) and is shown in Figure R 7(B), Part A. The other group was blotted onto nitrocellulose (Section M 5), probed with anti-Li's protein IgG antibodies (Section M 16) and the bound antibodies visualised with biotinylated protein A (Section M 9). The undigested protein was probed on a separate filter from the digestion products and the two subsequently aligned. The filters are shown in Figure R 7(B), Part B. The molecular weight markers were those described in Section M 2 for use in SDS-PAGE. The biotin-stained filters were overlaid with tracing paper, the bands drawn in pencil to improve clarity and the whole photographed. The positions of the molecular weight markers were recorded in pencil on the filters.

In each case 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gels were used.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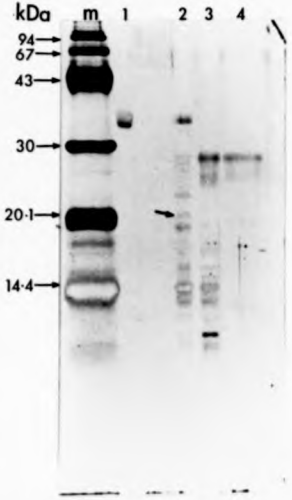
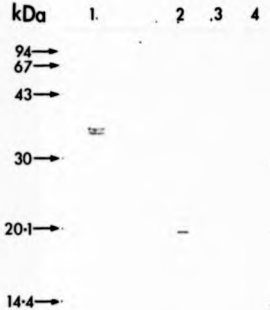
20·1
14·4

A




B



Validation of the Cross-Reaction Between Anti-Li's Protein Antibodies and the CRD.

The cross-reaction between anti-Li's protein antibodies and the CRD described in the previous section was assumed to be due to sequence homology between Li's protein and the CRD rather than the result of crystalloid contamination of the antigen used to raise anti-Li's protein antibodies. There are a number of observations which support this view of which the most important is probably the purity of the antigen. The antigen against which anti-Li's protein antibodies were raised was Li's protein cut from a sample of the 2S albumin fraction resolved on an SDS-polyacrylamide gel [see Materials and Methods, Section M 16]. Soluble castor bean proteins are readily purified away from the crystalloid proteins on the basis of the insolubility in water of the latter [Gifford et al., 1982]. The soluble protein was further purified by filtration through a Sephadex G-50 column, separating the 2S albumins from proteins of molecular mass greater than about 20 kDa [see Figure R 2(B), track 2]. Since the SDS-polyacrylamide gel used to resolve the 2S albumins was non-reducing any residual crystalloid protein would migrate as subunits of about 50 kDa, only degradation products of which could contaminate the 2S albumins. The other important feature of the cross-reaction is that it is specific for a single doublet out of the complex crystalloid fraction.

Another approach to validating the cross-reaction is to determine if the cross-reacting antibodies are directed against a restricted region of the CRD. The rationale behind this approach is that a cross-reaction based on contamination of the antigen by the CRD would probably give rise to antibodies directed against determinants over the length of

the protein. A cross-reaction based on sequence homology would probably be restricted to a discrete region of the protein. The method adopted for investigating the specificity of the cross-reaction was partial proteolysis of the reduced CRD and Western blot analysis of the products using anti-Li's protein antibodies.

The protease chosen was Staphylococcal V8 protease which cleaves specifically at the -COOH terminal side of glutamic or aspartic acids [Drapeau et al., 1972]. Initially conditions were established for the controlled proteolytic cleavage of the reduced CRD. The extent of proteolysis was varied by using a range of stock protease concentrations. Figure R 7(A) shows that the stock concentrations of V8 protease which gave a broad size range of digestion products were 5 μg/ml and 50 μg/ml [Figure R 7(A), tracks 3 and 4].

Using V8 protease at these stock concentrations the digestion products were blotted and probed with anti-Li's protein antibodies [Figure R 7(B)]. A single cleavage fragment of molecular mass 20.4 kDa reacted with the antibodies [Figure R 7(B), Part A, track 2, and Figure R 7(B), Part B, track 2]. This observation demonstrates that the cross-reaction between anti-Li's protein antibodies and the reduced CRD is restricted to a defined region of the crystalloid protein, although the 20.4 kDa fragment still represents 60% of the total mass of the reduced CRD.

Although consistent with the view that the cross-reaction is a reflection of sequence homology this result does not constitute conclusive proof. It may be argued, for example, that the cross-reacting fragment bears an immunodominant site, the only determinant sufficiently potent to elicit an IgG response when the crystalloid antigen is present at very low concentrations. On balance, though, the evidence is taken to indicate the presence of at least one antigenic determinant common to both Li's protein and the reduced CRD.

Figure R 7(C) Chemical cleavage of the reduced CRD

Four samples of the reduced CRD were excised from an SDS-polyacylamide gel as described in the legend to Figure R 7(A). The following groups of samples were resolved, in duplicate, on a second SDS-polyacrylamide gel:

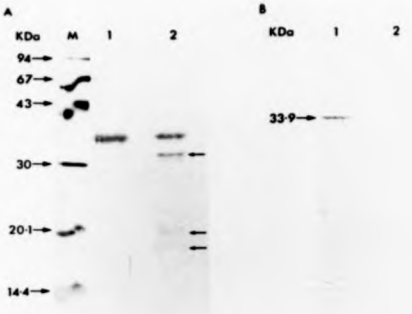
| | |
|---|---|
| Track 1 | reduced CRD, untreated |
| Track 2 | reduced CRD, treated with N-chlorosuccinimide |
| Track M | molecular weight markers |

One group was visualised by silver staining (Sub-section M 4(B)) and are shown in Figure R 7(C), Part A. The other group was blotted onto nitrocellulose (Section M 5) and probed with anti-Li's protein IgG antibodies. The bound antibodies were visualised using biotinylated protein A (Section M 9) and the filter is shown in Figure R 7(C), Part B.

In each case a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel was used.

Digestion with N-chlorosuccinimide was as described in Section M 10 and the molecular weight markers together with the assignment of their positions were as described in the legend to Figure R 7(B).

The arrows in Figure R 7(C), Part A indicate digestion products.


A
KDa
M
1
2
94
67
43
30
20·1
14·4
B
KDa
1
2
33·9

Chemical Cleavage of the Reduced CRD - An Anomalous Result.

In this study the main disadvantage to partial proteolysis was found to be a lack of reproducibility. It was found that despite attempts to standardise the digestion time and concentrations of protease and substrate, the pattern of cleavage products varied slightly between gels. Hence, in identifying the cross-reactive crystalloid fragment, samples from the same gel were used for both blotting and silver staining.

The reproducibility of at least one method of chemical cleavage, oxidative chlorination of tryptophanyl peptide bonds with N-chlorosuccinimide [Schechter et al., 1976], was found to be better than that of proteolysis. This method also generated fewer fragments [see Figure R 7(C), Part A]. Most of the protein, however, remained undigested despite attempts to increase the amount of protein cleaved by extending the reaction time.

Reduced CRD treated with N-chlorosuccinimide, including that which was undigested, failed to react with anti-Li's protein antibodies [Figure R 7(C), Part B]. This result was reproducible. It was concluded that N-chlorosuccinimide modified the protein in such a way as to abolish its reactivity with anti-Li's protein antibodies and that the modification specifically involved tryptophan residues. Schechter et al. [1976] noted that native proteins were not cleaved by N-chlorosuccinimide but that the indole ring of exposed tryptophan residues was oxidised to the oxindole species. Although, in this study, digestion was carried out in the presence of 6 M urea the protein may not have been fully denatured and, therefore, would be resistant to cleavage but subject to modification.

The abolition of cross-reactivity on treatment of the CRD with N-chlorosuccinimide is consistent with there being only one or a small number of antigenic determinants common to both Li's protein and the crystalloid doublet. To argue that the two proteins have a large number of common determinants would be to imply that tryptophan is important to the antigenicity of all of them.

It is unlikely that tryptophan is a component of the common antigenic determinant(s) as there are no tryptophan residues in the sequence of Li's protein [see Introduction, Figure I 1]. It is well established, though, that modification of amino acids close to an antigenic determinant can compromise the antigenic properties of that site [Atassi, 1978].

Of particular relevance are the early observations of Atassi and Thomas (1969) who found that specific modification of the arginine residues of sperm whale myoglobin resulted in a derivative having reduced immunological reactivity with antibodies raised against the unmodified myoglobin. Four fragments of the unmodified myoglobin were generated by cyanogen bromide cleavage, purified and the arginines of each specifically modified. The immunochemical reactivity of three of the fragments was unaffected by the modification while that of the fourth was significantly lowered. Specific modification of the single arginine residue of the fourth fragment was shown to account for the lowered immunological reactivity of the unfragmented, modified protein.

This unexpected effect on antigenicity has also highlighted one possible limitation to the use of protein fragments derived by chemical cleavage.

Figure R 7(D) The ability of barley and wheat storage proteins to bind anti-Li's protein IgG antibodies

Water soluble and water-insoluble protein was isolated from both wheat and barley (Section M 12(B)). 20 μg samples of each fraction were resolved as two groups, wheat proteins and barley proteins, on an SDS-polyacrylamide gel along with a 10 μg sample of castor bean crystalloids in each case. All samples were reduced (Section M 2): Figure R 7(D), Part A.

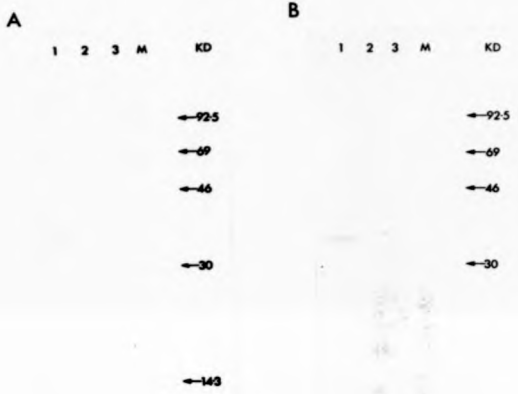
| | |
|---|---|
| Track 1 | castor bean crystalloids |
| Track 2 | water-soluble wheat proteins |
| Track 3 | water-insoluble wheat proteins |
| Track M | molecular weight markers |

Figure R 7(D), Part B.

| | |
|---|---|
| Track 1 | castor bean crystalloids |
| Track 2 | water-soluble barley proteins |
| Track 3 | water-insoluble barley proteins |
| Track M | molecular weight markers |

The two groups of protein were blotted onto nitrocellulose (Section M 5), probed with anti-Li's protein IgG antibodies and then with iodinated protein A (Section M 7). The filters were exposed to film for four days (Section M 23). The radiolabelled molecular weight markers are those described in Section M 2.

In each case a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel was used.


A
1 2 3 M KD
92·5
69
46
30
14·3
B
1 2 3 M KD
92·5
69
46
30

D 7(D)

The Crystalloid Cross-Reaction in the Context of Seed Storage Protein Evolution.

The cross-reaction between anti-Li's protein antibodies and the reduced CRD is interpreted as being an indication of sequence homology and, hence, of an evolutionary relationship. Recently a superfamily of seed storage proteins with limited sequence homology has been proposed [Kreis et al., 1985]. The superfamily is composed of proteins of diverse origins including; the prolamins of the Triticeae, the Bowman-Birk inhibitor of soybean, the trypsin inhibitor of barley and, importantly, the 2S albumin of castor bean and rape. As there is no sequence data for the crystalloids they could not be included in the comparison.

On the basis of its cross-reaction with anti-Li's protein antibodies the possibility existed that the CRD is a member of the superfamily proposed by Kreis et al. [1985]. To test this possibility extracts of water-soluble and water-insoluble protein were prepared from wheat and barley. The extracts were blotted and probed with anti-Li's protein antibodies. Figure R 7(D) [A + B] shows that none of the wheat or barley proteins are recognised by the antibodies. The antibodies did react with the CRD on both blots suggesting that the negative result was not due to technical problems in preparing and probing the filter.

The cross-reaction between anti-Li's protein antibodies and the CRD is not, then due to the sequence homology which forms the basis of the storage protein superfamily classification of Kreis et al. [1985]. It can further be concluded that the regions of sequence homology common to the superfamily members are not important antigenic determinants in Li's protein because anti-Li's protein antibodies did not recognise any of

the related wheat or barley proteins. For this reason it cannot be concluded that the crystalloid protein is not a member of the superfamily proposed by Kreis et al. [1985].

It is also possible that the CRD and Li's protein are members of another family of related seed storage proteins for which the superfamily of Kreis et al. [1985] serves as a precedent.

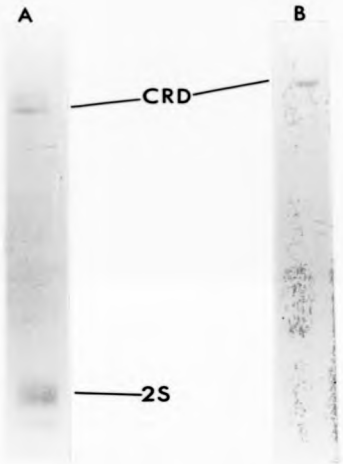
Figure R 7(E) The ability of the unreduced CRD to bind IgE from the CB pool

20 μg of unreduced crystalloid protein were resolved by SDS-PAGE (Section M 2). The unreduced CRD was excised, re-electrophoresed on a second SDS-polyacrylamide gel along with 20 μg of unreduced, total crystalloid protein.

The two protein samples were blotted onto separate nitrocellulose filters (Section M 5), probed with the CB pool and then with iodinated anti-IgE (Section M 8). The filters are shown in Figure R 7(E):

| | |
|---|---|
| Part A | unreduced crystalloid protein |
| Part B | unreduced CRD |

Traces of unreduced 2S albumins (2S) are visible in Track A.


A
B
CRD
2S

Significance of the Cross-Reactive Crystalloid Protein.

There is evidence to suggest that those features of a protein antigenic with respect to IgG synthesis are the same as those features antigenic with respect to IgE synthesis [Baur et al., 1986]. It is likely, therefore, that IgE antibodies directed against Li's protein will cross-react with the same crystalloid proteins as the anti-Li's protein IgG antibodies.

Of the three major IgE-binding proteins identified in Figure R 1, one is apparently a single band of molecular mass 34 kDa. The molecular weight of this putative allergen is closely comparable with that of the reduced CRD. The slight discrepancy can be accounted for by the different gel systems and calibration curves used to determine the respective molecular weights. The putative 34 kDa allergen may, therefore, be the same reduced crystalloid doublet which reacts with the IgG antibodies raised against Li's protein. In support of this suggestion the extreme insolubility in water of the crystalloid proteins would make them highly atypical allergens.

To test this idea a sample of total, unreduced crystalloid protein together with the unreduced CRD, which had been cut out of an SDS-polyacrylamide gel and re-electrophoresed, were probed with the CB pool [Figure R 7(E)]. In the track of total, unreduced crystalloid protein a single band with some smearing reacted with the CB pool [Figure R 7(E), Part A]. Contaminating 2S albumins were also visible. The isolated unreduced CRD reacted with the CB pool [Figure R 7(E), Part B]. Although there are no molecular weight markers it is clear that, since only one band in the total crystalloid fraction reacted with the CB pool, and since the isolated, unreduced CRD also reacts with the CB

pool, then the IgE-binding protein of the unreduced crystalloid fraction corresponds to the doublet recognised by the anti-Li's protein IgG antibodies.

Despite repeated attempts it has proved impossible to demonstrate an affinity between any component of the reduced crystalloid fraction and the CB pool. This may be due to insufficient protein on the blots or the technical difficulties involved in probing protein blots with immunoglobulins of such low abundance as IgE [about 200 ng/ml in normal serum; see Roitt, 1980]. To overcome this problem it will be necessary to directly measure the levels of specific IgE directed against the reduced CRD purified to homogeneity.

It is provisionally concluded, therefore, that the putative 34 kDa allergen corresponds to the reduced CRD and may not, in consequence, be a true allergen.

## Conclusions

The purity of the antigen used to raise anti-Li's protein IgG antibodies, the specificity of the cross-reaction between anti-Li's protein antibodies and the CRD and the fact that the cross-reaction is restricted to a specific fragment of the reduced CRD together strongly suggest that Li's protein and the CRD share some sequence homology.

That the chemical modification of a single amino acid, tryptophan, completely abolishes the antigenicity of the reduced CRD with respect to anti-Li's protein antibodies suggests that there is only a small number of common antigenic determinants, possibly only one.

There is a precedent for limited sequence homology between diverse types of seed storage proteins in the superfamily of Krais et al. [1985] of which Li's protein is a member. There is no evidence to suggest that the CRD is a member of this superfamily. The amino acid sequences which form the basis of the superfamily classification are not important antigenic determinants for Li's protein.

IgE from the CB pool reacts with the unreduced CRD although reduced CRD fails to react with the CB pool which may be due to technical difficulties. It is possible, therefore, that the putative 34 kDa allergen is the cross-reactive, acidic crystalloid polypeptide and, as such, is not a true allergen.

# SECTION 8

## Purification of the Reduced CRD.

## Summary of Results

The reduced CRD was substantially purified by ion-exchange chromatography. Silver staining of the partially purified reduced CRD revealed that the upper band of the reduced CRD doublet, as visualised by Coomassie staining, may be composed of two polypeptides.

A preliminary attempt was made to separate and completely purify the individual CRD polypeptides by chromatofocusing.

Figure R 8(A) Determining the optimum binding conditions for the reduced CRD to DEAE-Sephacel

The details of the experimental protocol are given in Section M 14. Five samples of 50-100 mg of crystalloid protein were dissolved in binding buffer at five different pH values: 5.0, 6.0, 7.0, 8.0 and 9.0. Each sample was incubated with DEAE-Sephacel which had been equilibrated in binding buffer of the corresponding pH. The bound and unbound protein in each case was collected and analysed by SDS-PAGE using a 15 (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel.

Labelling of Figure R 8(A):

Numbers indicate pH values: 5.0, 6.0, 7.0, 8.0 and 9.0.

Letters indicate unbound protein (s), bound protein (b), total, reduced crystalloid protein (t) and molecular weight markers (m). The molecular weight markers were those described in Section M 2 for use in SDS-PAGE. The proteins were stained with Coomassie blue (Sub-section M 4(A)).

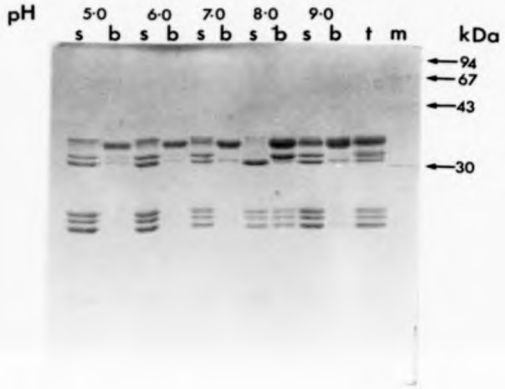

pH
5·0
6·0
7·0
8·0
9·0
s b s b s b s b s b t m
kDa
94
67
43
30

Figure R 8(B) Elution profile of reduced crystalloid protein fractionated by DEAE-Sephacel column chromatography

Reduced crystalloid protein isolated from 15 g of beans (Subsection M 12(A)) was applied to a DEAE-Sephacel column (2.5 x 15 cm) and bound protein eluted with a 0-0.15 M NaCl gradient. The arrows in Figure R 8(B) indicate the beginning and the end of the gradient. Protein still bound to the column was eluted with a column volume of 0.5 M NaCl. Two ml fractions were collected and the absorbance at 280 nm of even numbered fractions measured and plotted against fraction number. The conductivity of every tenth fraction was measured and the corresponding sodium chloride concentration plotted against fraction number.

A 280 (·)
Fraction Number
NaCl (mM) -o-

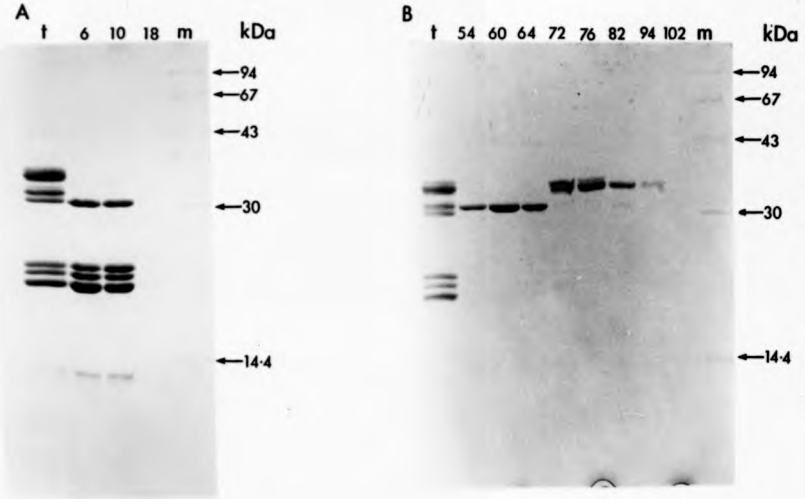

A
t 6 10 18 m
kDa
94
67
43
30
14·4
B
t 54 60 64 72 76 82 94 102 m
kDa
94
67
43
30
14·4

Figure R 8(C) SDS-PAGE analysis of the absorbance peaks shown in Figure 8(B)

Aliquots of fractions from each $A_{280}$ peak shown in Figure R 8(B) were analysed by SDS-PAGE using a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel.

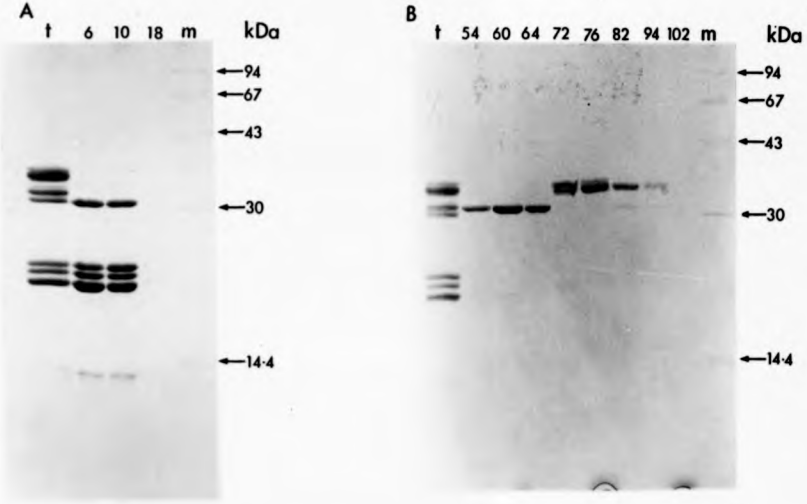
Part A

| Fraction number | µl |
|---|---|
| 6 | 30 |
| 10 | 20 |
| 18 | 20 |

Part B

| | |
|---|---|
| 54 | 30 |
| 60 | 15 |
| 64 | 30 |
| 72 | 15 |
| 76 | 8 |
| 82 | 10 |
| 94 | 30 |
| 102 | 30 |

Molecular weight markers (m) were those described in Section M 2.
Samples of total crystalloid protein (t) were prepared as described in Sub-section M 12(A).
All samples were reduced (Section M 2).


A
t 6 10 18 m kDa
94
67
43
30
14·4
B
t 54 60 64 72 76 82 94 102 m kDa
94
67
43
30
14·4

Partial Purification of the Reduced CRD by Ion-Exchange Chromatography.

Rigorous purification of the reduced CRD is necessary to allow direct, quantitative measurement of the IgE response against this protein. In this way it is possible to determine if the reduced CRD is the 34 kDa putative allergen as suggested in the preceding section.

A partial purification of unreduced crystalloid subunits had previously been achieved using ion-exchange chromatography [Gifford and Bewley, 1983a]. This technique was adopted for the purification of the reduced CRD.

As the reduced CRD is acidic an anion-exchanger, DEAE-Sephacel, was chosen. The binding properties of the reduced acidic polypeptides over a range of pH values was investigated. It was found that the reduced CRD was bound most efficiently in the pH range 6-7 with the least degree of contamination from other proteins judged to be at pH 7.0 [see Figure R 8(A)].

Consequently the acidic crystalloid polypeptides were bound to a DEAE-Sephacel column at pH 7.0 and eluted with a sodium chloride gradient [Figure R 8(B)]. SDS-PAGE analysis of the eluted protein showed that not only did the basic polypeptides fail to bind but neither did one of the 30 kDa acidic polypeptides [Figure R 8(C), Part A]. This unbound 30 kDa polypeptide is probably the least acidic of the group.

The reduced CRD started to elute as part of the second gradient peak [Figure R 8(B)]. SDS-PAGE analysis of sequential samples in the second peak showed that it was not homogeneous; the leading edge was composed of qualitatively equal amounts of the CRD and a protein of higher molecular weight [Figure R 8(C), Part B, track 72], which

disappeared towards the trailing edge of the peak [Figure R 8(C), Part B, track 94]. This is in contrast to the first peak of the gradient [Figure R 8(B), fractions 50 to 68] which is homogeneous in composition [Figure R 8(C), Part B, tracks 54, 60 and 64].

Figure R 8(D) Coomassie blue staining and silver staining of the partially purified, reduced CRD

The partially purified, reduced CRD, fractions 92-108 in Figure R 8(B), were pooled and dialysed as described in Section M 14. A five μg sample of the protein was resolved using SDS-PAGE (Section M 2) and stained with Coomassie blue (Sub-section M 4(A)):

Part A

| | |
|---|---|
| Track 1 | total crystalloid protein |
| Track 2 | partially purified CRD |
| Track 3 | molecular weight markers |

A sample of approximately 0.5 μg was also resolved using SDS-PAGE and silver stained:

Part B

| | |
|---|---|
| Track 1 | partially purified CRD |
| Track M | molecular weight markers |

Molecular weight markers are those described in Section M 2 for use in SDS-PAGE.

All samples were reduced (Section M 2).
A 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving gel was used in both cases.

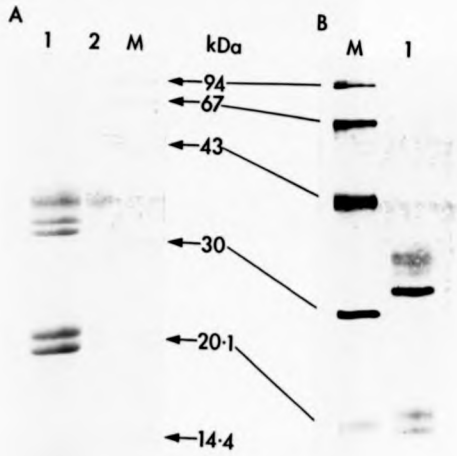
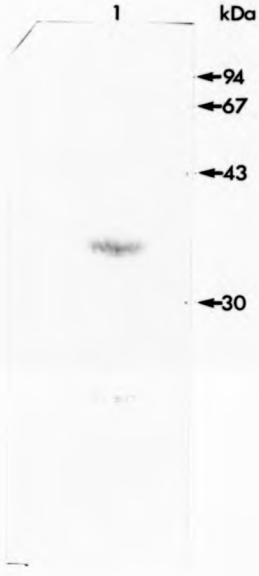

A
1
2
M
kDa
94
67
43
30
20·1
14·4
B
M
1

Figure R 8(E) Western blot analysis of the partially purified, reduced CRD

A five μg sample of the partially purified, reduced CRD was resolved on a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide resolving polyacrylamide gel (Section M 2) and blotted onto nitrocellulose (Section M 5). The filter was probed with anti-Li's protein IgG antibodies and the bound protein visualised using biotinylated protein A (Section M 9).

Molecular weight markers for use in SDS-PAGE (Section M 2) were blotted from the same gel onto a separate strip of nitrocellulose and stained with Amido black (Section M 5). The positions of the markers were recorded in pencil on the nitrocellulose strip bearing the CRD.


1
kDa
94
67
43
30

Analysis of Partially Purified, Reduced CRD by SDS-PAGE and Western Blotting.

Fractions 82 to 102, inclusive, were pooled, dialysed against water and freeze-dried in preparation for further purification by chromatofocusing. Samples of the partially purified CRD were analysed by Coomassie blue and silver staining using SDS-PAGE [Figure R 8(D)]. Coomassie staining revealed traces of basic polypeptides at about 20 kDa [Figure R 8(D), Part A]. Silver staining, though, intensely stained a 32 kDa trace contaminant so that it appeared to be the major component of the fraction [Figure R 8(D), Part B]. Considered in conjunction with the problems experienced in trying to silver stain Li's protein [Results and Discussion, Sub-section D 3(E)] it is clear that considerable caution should be exercised in interpreting silver stained gels of complex protein mixtures.

Figure R 8(D), Part B also shows that the reduced CRD is composed of a doublet of two equally intense protein bands. This band pattern is not the same as that seen on Coomassie staining of the reduced CRD which appears as a doublet composed of a thick upper band and a thin lower band [see Figure R 6(E)]. It is possible that the thick upper band of the reduced CRD doublet, as seen on Coomassie staining, is, itself, composed of two bands.

Western blot analysis of the partially purified, reduced CRD probed with anti-Li's protein antibodies confirmed that the correct protein had been purified [Figure R 8(E)].

Figure R 8(F) Elution profile of the reduced CRD fractionated on a chromatofocusing column

Half of the partially purified, reduced CRD was applied to a 10 ml column of the anion exchanger PBE 94 and eluted with polybuffer as described in Section M 14. The end of the pH gradient is indicated by an arrow in Figure R 8(F). Two ml fractions were collected and the absorbance at 280 nm of the even numbered fractions measured and plotted against fraction number.

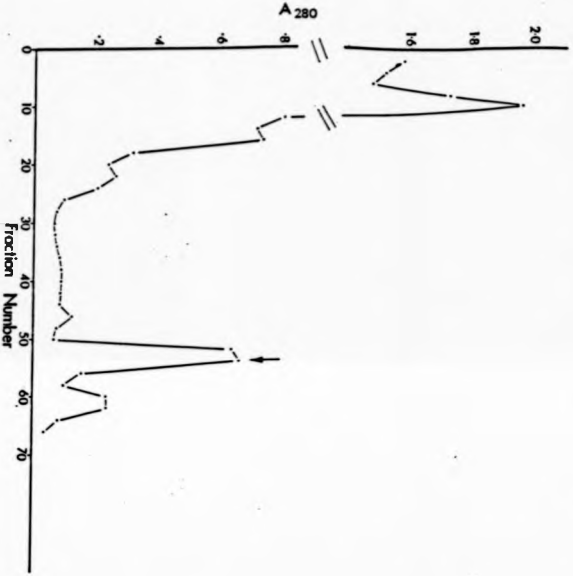

A 280
0
2
4
6
8
16
18
20
10
20
30
40
50
60
70
Fraction Number

Figure R 8(G) SDS-PAGE analysis of the protein peaks shown in Figure R 8(G)

50 µl aliquots of the $A_{280}$ peaks shown in Figure R 8(F) were resolved on a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide SDS polyacrylamide gel (Section M 2) and silver stained (Sub-section M 4(B)).

The molecular weight markers are those described in Section M 2 for use in SDS-PAGE.

The gel was smaller than those routinely used in this study, 17 x 17 x 0.15 cm.

The number of a track corresponds to the number of the fraction (Figure R 8(F)) analysed in that track.

All samples were reduced.

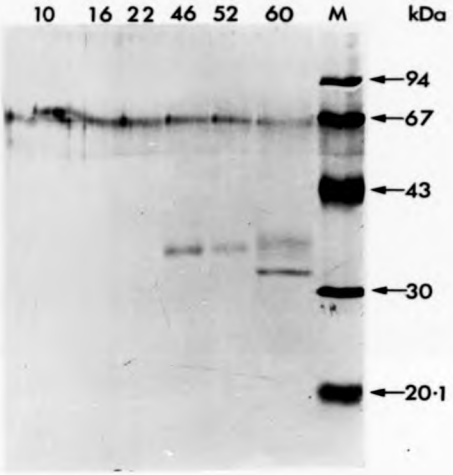

10
16
22
46
52
60
M
kDa
94
67
43
30
20·1

Table R 2 The pH value of every tenth fraction eluted from the chromatofocusing column used to fractionate the reduced CRD

Reduced CRD which had been partially purified using ion-exchange chromatography was fractionated using a chromatofocusing column (Section M 14). Table R 2 presents the approximate pH values of every tenth fraction eluted from the chromatofocusing column (Figure R 8 (F)). The pH values were estimated using indicator paper.

| Fraction Number | pH |
|---|---|
| 1 | 7.0 |
| 10 | 7.0 |
| 20 | 6.0 |
| 30 | 6.0 |
| 40 | 5.0-5.5 |
| 50 | 5.0 |

Chromatofocusing as a Technique for the Complete Purification and Separation of the Reduced CRD Proteins.

Chromatofocusing was chosen as a technique for the purification of the individual CRD polypeptides since it relies on differences in charge rather than size, is capable of a high degree of resolution and is simple to use [see Pharmacia booklet, "Chromatofocusing"]. As in the case of ion-exchange chromatography, chromatofocusing had to be performed under unusual buffer conditions. The insolubility of the crystalloid proteins in water meant that the column had to be run in a high concentration of urea. To prevent possible aggregation of the crystalloid polypeptides a reducing agent was required, in this case 2-mercaptoethanol [2-ME]. The possible effects of a strong reducing agent on the polybuffer eluent were unknown, also the 2-ME quickly turned brown on exposure to air making accurate $A_{280}$ measurements of small amounts of protein very difficult. A compromise was achieved by pre-washing the column in buffer made up to a 2-ME concentration of 5% [v/v] and by loading the protein in buffer at the same concentration of 2-mercaptoethanol. The polybuffer, though did not contain 2-ME.

The ability of 2-ME to absorb strongly at 280 nm explains the very high $A_{280}$ readings obtained over the first 25 fractions [Figure R 8(F)]. The $A_{280}$ of the start buffer which contained 2-ME and which was used to pre-wash the column was 1.$\not$8 absorbance units. In consequence, although the peak centred around fraction 10 was shown to contain protein [see Figure R 8(G), track 10], the contribution of the protein to the total $A_{280}$ was probably small.

Proteins of the Reduced CRD Purified by Chromatofocusing.

Figure R 8(G) shows that four proteins were purified by chromatofocusing [Figure R 8(G), tracks 10, 46, 52 and 60]. These proteins corresponded to the peaks of the elution profile [Figure R 8(F)]. The fraction 10 peak contained a large protein of molecular mass 71 kDa. This protein was not seen in the silver stained sample of CRD nor has a band of comparable size been seen in samples of total crystalloid protein, reduced or unreduced. It is also noteworthy that coincident with the appearance of a novel crystalloid band there is no trace of the contaminating 20 kDa basic polypeptides [Figure R 8(D), Part B].

One possible explanation for the appearance of the 71 kDa band is that, in the absence of a sufficiently strong reducing environment, the 20 kDa basic subunits aggregated. As 71 is not an integral multiple of 20 the 71 kDa band cannot be composed solely of basic polypeptides. A model in which two 20 kDa polypeptides combine with one acidic polypeptide of about 30 kDa would give a multimer of the required molecular weight. The inclusion of an acidic polypeptide would probably be essential to aggregation in helping to counteract the charge repulsion between basic polypeptides. The 71 kDa protein cannot be composed solely of acidic polypeptides as it elutes at about pH 7.0 [Table R 2].

A simpler explanation for the absence of the basic polypeptides is that the anionic column repelled them and they were quickly eluted. As none of the column fractions before number 10 were analysed by SDS-PAGE this idea cannot be conclusively refuted. There is, however, no indication of a protein peak before that centred around fraction number

10.

That the 71 kDa aggregate remains associated on SDS-PAGE, having been treated with dithiothreitol [DTT] prior to loading onto the gel, may also be explained in terms of an inadequate reducing environment. Crystalloid samples for SDS-PAGE have been routinely reduced by adding one $\mu$l of a 0.5 M stock solution of DTT to a sample volume not greater than 30 $\mu$l. In this case, though, the sample volume was 100 $\mu$l giving an effective DTT concentration of less than one third that usually employed.

Of the other proteins purified fraction 60 contains the 32 kDa contaminant together with traces of the reduced CRD doublet. Fractions 46 and 52 show two pure proteins, that in fraction 46 having a slightly lower molecular weight than that in fraction 52. The apparent difference in molecular weights between the two proteins is small and may be artefactual, the result of slightly different migration rates between tracks. That the two proteins eluted as separate peaks suggests, though, that they are different proteins.

The two proteins of fractions 46 and 52 may be the individual polypeptides comprising the reduced CRD doublet visualised by silver staining [Figure R 8(D), Part B]. It should be noted that, compared to the traces of the reduced CRD doublet in track 60, the protein in fraction 52 migrates with the lower band of the doublet while the protein in track 46 migrates faster than either of the doublet bands. If the reduced CRD doublet visualised by silver staining is really the upper band of the doublet visualised by Coomassie staining then the protein in track 46 may be the lower band of the Coomassie-stained doublet. There is no trace of the purified upper band of the silver stained doublet.

Another striking feature of Figure R 8(G) is the 66 kDa band in every track. A high molecular mass band is often seen on silver-stained

gels but generally runs at about 54 kDa; traces of this band can be seen in Figure R 8(G). Like the 54 kDa band the 66 kDa band is probably a feature of the gel rather than of the protein samples since it can be seen in the edge of the track, immediately to the left of track 10, which contained no protein.

This preliminary experiment suggests that chromatofocusing will be of use in clarifying the polypeptide composition of the reduced CRD and in purifying its component polypeptides. The conditions for purification must be more rigorously defined, though, in particular the minimum concentration of 2-ME required to fully reduce the crystalloid complex. The pH gradient did not completely form in this experiment, reaching only pH 5.0 instead of pH 4.0 [see Table R 2].

## Conclusions

The upper band of the reduced CRD, as visualised by Coomassie staining, may be composed of two polypeptides.

The combination of anion-exchange chromatography and chromatofocusing forms the basis of a purification protocol capable of resolving the individual polypeptides of the reduced CRD.

SECTION 9

# Identification of the Precursor to Li's Protein and Studies on the Synthesis of Castor Bean cDNA.

## Summary of Results

*In vitro* translation of castor bean messenger RNA and immunoprecipitation with anti-Li's protein antibodies identified a 34 kDa polypeptide as the precursor to Li's protein. Li's protein precursor was shown to be identical to a previously characterised 2S albumin precursor.

The RNAse H method for cDNA synthesis produced first strand but not second strand castor bean cDNA. The inability to synthesise second strand was restricted to castor bean cDNA.

The S1 nuclease method for cDNA synthesis produced both first and second strand castor bean cDNA. The synthesis of first strand cDNA using the S1 nuclease method was almost twice as efficient as that using the RNAse H method.

Background to the Identification of the Precursor to Li's Protein.

A 2S storage albumin precursor had previously been identified by Rutterworth and Lord [1983]. They demonstrated that, in the absence of microsomes, antibodies raised against a crude 2S albumin fraction precipitated a 34 kDa protein which was N-terminally cleaved to a molecular mass of 32.5 kDa in the presence of microsomes. Further, the 32.5 kDa protein was shown to be transiently associated with the endoplasmic reticulum and to yield mature proteins in the 2S albumin molecular mass range [about 10 kDa] which were deposited in the protein body matrix. It was concluded that the 32.5 kDa protein was a 2S albumin precursor.

In the absence of a reducing agent the 2S albumin precursor migrated with an apparent molecular mass of 22 kDa using SDS-PAGE. The explanation proposed for this unusual behaviour was that a large number of intra-chain disulphide bonds maintained the protein in a compact, globular structure; an explanation supported by the high cysteine content of the 2S albumin fraction [Youle and Huang, 1978]. The 2S albumin precursor had previously been shown to be unglycosylated [Roberts and Lord, 1981a]. The exact identity of the 2S albumin proteins against which antibodies had been raised was not determined.

Figure R 9(A) Immunoprecipitation of the protein precursor to Li's protein

In-vitro translation of castor bean message isolated from developing beans at stages D-E (Roberts and Lord, 1980) was carried out as described in Section M 18. Immunoprecipitation using anti-Li's protein antibodies was also as described in Section M 18.

The following samples were resolved on a 10% (w/v) acrylamide, 0.26% (w/v) bisacrylamide SDS-polyacrylamide gel:

Track 1 endogenous translation products, unreduced.

Track 2 endogenous translation products, reduced.

Track 3 castor bean message translated in the absence of membranes, immunoprecipitate unreduced.

Track 4 castor bean message translated in the absence of membranes, immunoprecipitate reduced.

Track 5 castor bean message translated in the presence of membranes, immunoprecipitate unreduced.

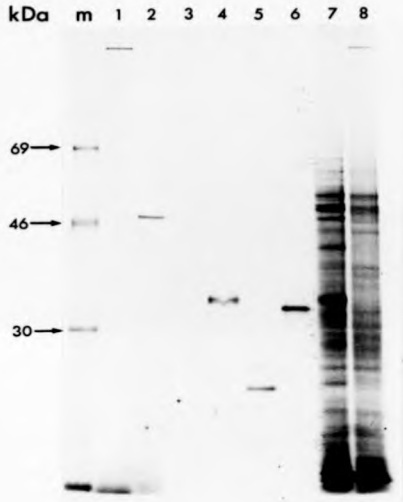
Track 6 castor bean message translated in the presence of membranes, immunoprecipitate reduced.

Track 7 castor bean message, total translation products reduced.

Track 8 castor bean message, total translation products unreduced.

The membranes used were dog pancreatic microsomes (Section M 18). Radiolabelled molecular weight markers were as described in Section M 2.

The gel was fluorographed (Section M 19), dried (Section M 2) and exposed to film for two weeks (Section M 23).


kDa
m
1
2
3
4
5
6
7
8
69
46
30

Identification of the Precursor to Li's Protein.

In the present study immunoprecipitation with anti-Li's protein antibodies was used to determine if the precursor to Li's protein is the 2S albumin precursor characterised by Butterworth and Lord [1983]. *In vitro* translations in the absence of membranes [Figure R 9(A), track 4] and in the presence of membranes [Figure R 9(A), track 6] and subsequent immunoprecipitation identified a 34 kDa protein which was N-terminally cleaved to a molecular mass of 32.5 kDa. Identical immunoprecipitations but without reducing the protein prior to SDS-PAGE analysis showed that the 32.5 kDa protein migrated with an apparent molecular mass of 22 kDa [Figure R 9(A), track 5]. The precursor translated in the absence of membranes, retaining its signal sequence, and which was not reduced prior to SDS-PAGE did not immunoprecipitate [Figure R 9(A), track 3]. This result is probably an artefact resulting from the combination of two factors; the poor translation efficiency of that sample as assayed by $^{35}$S-methionine incorporation and the fact that the uncleaved precursor has been shown to be less antigenic with respect to anti-2S albumin antibodies than after N-terminal cleavage [Roberts and Lord, 1981b].

On the basis of its size and its unusual mobility properties the precursor precipitated by anti-Li's protein antibodies was identified as that previously characterised by Butterworth and Lord [1983]. The true molecular weight of the precursor is likely to be that estimated under reducing conditions and, as such, the precursor is about three times larger than the mature protein [molecular mass 11 kDa]. This large size discrepancy can be accounted for in a number of ways. Much of the precursor may not be included in the final product; a precedent for this

idea is found in the case of a napin precursor. Napins are small, basic, heterodimeric seed storage proteins of oilseed rape, Brassica napus. Crouch et al. [1983] isolated and sequenced the precursor to a napin storage protein and showed that 35% of the precursor sequence was not found in the mature protein. Alternatively, the precursor may contain more than one copy of each Li subunit type or more than one member of the 2S albumin fraction. The latter idea is unlikely since the other members of the 2S albumin fraction are immunologically unrelated to Li's protein [Figure R 6(C)].

Despite the well characterised cross-reactivity between anti-Li's protein antibodies and at least one crystalloid protein, a crystalloid precursor was not immunoprecipitated. The crystalloid complex precursors have previously been identified as a group in the 46-55 kDa molecular mass range [Lord, 1985]. It was concluded that the determinant common to Li's protein and the crystalloid protein was not recognised in the crystalloid precursor.

As noted above Roberts and Lord [1981b] found that 2S albumin precursor was more antigenic after N-terminal cleavage, a result also seen in this study [compare tracks 4 and 6 of Figure R 9(A)]. The difference in antigenicity probably results from the N-terminally cleaved precusor conformation more closely resembling that of the mature protein than does the uncleaved precusor conformation. These observations further emphasise the importance of three-dimensional conformation to the antigenicity of Li's protein.

The identification of the precursor to Li's protein opens the way to the isolation of a corresponding cDNA clone.

D 9(D)

A Stage in Castor Bean Development Enriched for the Messenger RNA Encoding Li's Protein.

Investigation of the protein biosynthetic capacity of castor bean endosperm during development has shown that synthesis of the precursor to Li's protein is initiated and proceeds quickly at the onset of testa formation [Roberts and Lord, 1981a]. Messenger RNA [mRNA] isolated from this stage of development was used as the starting material for cDNA synthesis.

The assumption behind this choice is that the appearance of the precursor to Li's protein reflects the accumulation of the corresponding mRNA. The general form of the assumption has been verified for other seed storage proteins; the hordeins of barley [Miflin et al., 1984] and the 11S globulin of sunflower [Allen et al., 1985], and so is considered to be justifiable in this context. In neither of the examples cited can transcriptional regulation of gene expression be inferred since the levels of the specific nuclear transcripts were not measured.

The RNAse H and S1 Nuclease Techniques for cDNA Synthesis.

The synthesis of double-stranded cDNA proceeds by first synthesising the strand complementary to the mRNA, first strand, using reverse transcriptase, an RNA- or DNA- directed DNA polymerase [Kornberg, 1980]. First strand synthesis is primed by an oligo (dT) molecule annealed to the polyadenylated mRNA.

Anti-complementary or second strand cDNA is synthesised by DNA polymerase I [Dpol I]. The original approaches to cDNA synthesis involved self-priming of second strand synthesis by means of a hair-pin loop formed at the 3' end of the first strand. The hair-pin loop was subsequently degraded by S1 nuclease, a single-strand specific endonuclease [Ando, 1966]. The principal limitation to the technique is the S1 nuclease which often degrades part of the cDNA corresponding to the 5' end of the message [Murray et al., 1983].

The S1 nuclease technique had previously been used to synthesise castor bean cDNA [Lamb et al., 1985]. In the present study a more recent cDNA synthesis technique, not involving S1 nuclease, was tested. First strand synthesis utilises reverse transcriptase as previously described but second strand synthesis depends on nicking and degradation of the mRNA annealed in the RNA: DNA duplex. The RNA is degraded by RNAse H, an RNAse specific for RNA annealed to DNA [Hausen and Stein, 1970]. As the RNA is nicked and partially degraded so Dpol I is able to synthesise second strand DNA.

Figure R 9(B) Alkaline agarose gel analysis of cDNA synthesised using the RNAse H and S1 nuclease methods

Double stranded cDNA was synthesised from stage D-E castor bean mRNA (Roberts and Lord, 1980) using the S1 nuclease and RNAse H methods as described in Section M 20. Aliquots of the first strand, second strand and S1 nuclease-treated cDNA corresponding to 10 cps, 5 cps and 5 cps, respectively, were analysed on a 1.2% alkaline agarose gel as described in Section M 21. An aliquot of first strand cDNA synthesised using the RNAse H method and corresponding to 10 cps was also analysed on a 1.2% alkaline agarose gel. The gels were dried and exposed to film for three days (Section M 23). Figure R 9(B), Part A:

| | |
|---|---|
| Track 1 | First strand cDNA, RNAse H method |

Figure R 9(B), Part B:

| | |
|---|---|
| Track 1 | First strand cDNA, S1 nuclease method |
| Track 2 | Second strand cDNA, S1 nuclease method |
| Track 3 | S1 nuclease-treated double stranded cDNA |

The molecular size markers were the Hind III digestion products of phage lambda DNA: 23.13 Kb, 9.42 Kb, 6.56 Kb, 4.36 Kb, 2.32 Kb, 2.03 Kb, 0.56 Kb and 0.125 Kb.

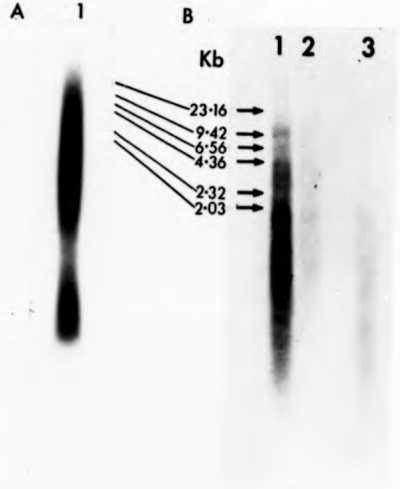

A
1
B
Kb
1
2
3
23·16
9·42
6·56
4·36
2·32
2·03

Table R 3 The Efficiencies of First and Second Strand cDNA Syntheses Using the S1 Nuclease Method and the RNAse H Method

cDNA was synthesised from castor bean mRNA using the S1 nuclease method and the RNAse H method as described in Sub-section M 20(B) and Sub-section M 20(C), respectively. Table R 3 presents the masses of first and second strand cDNA synthesised using each method and the synthetic yield for each synthesis. The synthetic yield is the mass of cDNA synthesised, expressed as a percentage of the mass of the template (Sub-section M 20(E)).

| | RNAse H Method | S1 Nuclease Method |
|---|---|---|
| μg mRNA (x) | 1.0 | 1.0 |
| μg first strand cDNA synthesized (y) | 0.098 | 0.162 |
| Synthetic yield $(\frac{y}{x} \cdot 100)$ | 9.8 | 16.2 |
| μg first strand cDNA (y) | 0.098 | 0.162 |
| μg second strand cDNA synthesized (z) | - | 0.024 |
| Synthetic yield $(\frac{z}{y} \cdot 100)$ | - | 15.0 |

Comparison of the S1 Nuclease and RNAse H Protocols for the Synthesis of Castor Bean cDNA.

The synthetic yield of first strand cDNA produced using the RNAse H method was low, 9.8% expressed as a percentage of the total possible yield. In comparison first strand synthesis using the S1 nuclease protocol was 16.2% efficient. Second strand synthesis using the S1 nuclease protocol had a 15% synthetic yield and completely failed using the RNAse H protocol. These results are summarised in Table R 3. The failure of second strand synthesis using the RNAse H protocol was a reproducible result and only occurred with castor bean mRNA; the same protocol was successfully used to synthesise second strand pea and wheat cDNA by other workers. This observation may indicate that castor bean mRNA is resistant to degradation by RNAse H or that there is an inhibitor of RNAse H in the mRNA sample. In this respect it is noteworthy that the castor bean mRNA samples used in this study had a light brown colour probably due to testa pigments carried through during the purification.

Alkaline agarose gel analysis of the first strand cDNA synthesised using the S1 nuclease and RNAse H protocols revealed the majority of the cDNA to be in the 0.5 Kb to 2.0 Kb molecular size range [Figure R 9(B)]. There was a significant proportion of high molecular size [2.0-10.0 Kb] first strand cDNA synthesized using the S1 nuclease protocol. In both cases distinct bands were visible, probably representing highly abundant mRNA species.

Although faint the S1 second strand cDNA profile is seen to be of a comparable molecular size range to that of the first strand [Figure R 9(B), Part B, track 2]. Treatment with S1 nuclease lowers the average

molecular size [compare track 3 with track 1 of Figure R 9(B), Part B probably because of degradation of the cDNA corresponding to the 5' end of the messenge as previously discussed. Despite this drawback the S1 nuclease method is clearly preferable to RNAse H since the latter fails to synthesise second strand.

## Conclusions

The precursor to Li's protein is the 2S albumin precursor previously characterised by Lord and co-worker.

The RNAse H method is ineffective in synthesising second strand castor bean cDNA. The S1 nuclease method should be used in constructing a castor bean cDNA library.

# SECTION 10

## Overall Summary of Results and Conclusions.

There are at least four proteins which may be castor bean allergens. One is a 12 kDa component of the 2S storage albumin fraction, Li's protein. Li's protein is an unglycosylated trypsin inhibitor composed of a large and a small subunit and is immunologically unrelated to the other 2S albumins. There is evidence to suggest that only the large subunit of Li's protein is allergenic. Li's protein may associate under non-denaturing conditions, a property which may be important in eliciting an IgE response. The antigenicity and, probably, allergenicity of Li's protein depends on the integrity of its three-dimensional conformation. Li's protein may be encoded by a multigene family and is initially translated as a 34 kDa precursor, almost three times its mature size. The physical and chemical characteristics of Li's protein are typical of many protein allergens. The identification of a 2S storage albumin as a putative castor bean allergen is entirely consistent with previous work in this area.

IgG antibodies raised against Li's protein strongly cross-react with two or three acidic crystalloid polypeptides of molecular mass about 34 kDa. One or more of the cross-reactive crystalloid polypeptides may be the 34 kDa putative castor bean allergen. In support of this idea IgE from patients allergic to castor beans binds to a single crystalloid subunit, that containing at least one of the cross-reactive acidic polypeptides.

The other putative castor bean allergens, two proteins of molecular mass about 50 kDa, have not been identified or isolated. This study has concentrated on endosperm proteins and so the unidentified allergens may be located in the seed testa.

For the synthesis of double-stranded castor bean cDNA the S1 nuclease method is preferable to the RNAse H method since the latter is ineffective in synthesising second strand castor bean cDNA.

A number of questions remain to be answered in order to present a more coherent picture of the allergenic components of the castor bean. The possibility that one or more of the 34 kDa crystalloid polypeptides is the putative 34 kDa allergen should be tested by quantitatively measuring the level of specific IgE from the sera of patients allergic to castor beans. The contributions of the large and small subunits to the allergenicity of Li's protein should be determined again by quantitative measurement of the IgE response to each. Finally the identity of the 50 kDa doublet should be established, concentrating on the proteins of the seed testa.

# CHAPTER 4

# CONCLUDING CHAPTER:
# THE USE OF PURIFIED ALLERGENS IN IMMUNOTHERAPY

## The Aims of Modern Allergen Research

Isolation and characterisation of a large number of protein allergens has failed to reveal any common physicochemical features which can account for the unique biological activity of this group of proteins. Other factors, including the route and dose of administration of the allergen and the genetic background of the individual, have been shown to be important in eliciting a hypersensitive response (see Sub-section I 3(E)).

Historically, the analyses of protein allergens have only taken into account their gross physicochemical properties such as amino acid sequence and isoelectric point. More detailed analyses, particularly in defining three-dimensional conformation and the location of antigenic determinants, may reveal structural features common to the whole range of protein allergens. Allergens of low molecular mass which are structurally simple should be particularly amenable to investigation and modification, a point illustrated by the large body of work describing the properties of the low molecular mass ragweed allergen, Ra5 (molecular mass five kDa).

A likely practical application of information obtained from the detailed structural and functional characterisation of a range of protein allergens will be to devise strategies for the prevention or suppression of allergic disease. At the very least, purified, characterised allergens are useful tools in elucidating the role of the extraneous factors mentioned above which are important in allergic disease.

The aim of this chapter is to briefly describe current approaches to immunotherapy, particularly those which involve purified, characterised allergens.

Hyposensitisation Therapy

An early approach to the treatment of allergic disease was hyposensitisation therapy: the induction of immunological unresponsiveness in a previously allergic subject using sequential, incremental inoculations of the native allergen. The most successful example of this approach has been the treatment of allergy to bee venom (Hunt et al., 1976), although the mechanism of protection is still not clear (Kemeny et al., 1983b). The induction of tolerance is associated with an increase in the levels of IgG to venom allergens (Hoffman et al., 1981). IgG antibodies produced during immunotherapy have been shown to be capable of blocking leucocyte histamine release in vitro (Lichtenstein et al., 1968) and reducing the reaction to bee venom in vivo (Lessof et al., 1977).

The IgG response during hyposensitisation is restricted to the IgG4 sub-class (Aalberse et al., 1983), and Urbanek et al. (1986) have shown that elevated levels of IgG4 specific for bee venom allergens are associated with long-term protection against allergic response to bee stings. It is believed that IgG4 serves a blocking function, binding to allergen before it can combine with immobilised IgE and precipitate an allergic reaction. Although attractive in its simplicity, this approach to immunotherapy has a number of weaknesses particularly in that it may not be generally applicable since bee venom allergy is atypical both in the relatively large amount of allergen administered at each exposure (see Sub-section I 3(C)) and in the route of administration. It should be noted, though, that in a study of schistosome-infected patients the relative contribution of IgG4 to the total immune response increased with the duration of the infection (Iskander et al., 1981). Another

atypical feature of bee venom allergy is that it does not seem to be confined to those atopic individuals genetically predisposed to allergy (Settipane and Chaffe, 1979).

An obvious problem in the use of native venom allergens in immunotherapy is that highly sensitised subjects may react adversely to even minute doses. In addition, since crude bee venom is usually used as antigen during hyposensitisation, IgE antibodies to additional bee venom allergens sometimes appear during therapy suggesting that a better approach may be to use purified venom allergens (Kemeny et al., 1983b).

## Antigen-Specific T-cell Suppression

Another approach to the prevention and control of immediate hypersensitivity is suppression of the IgE response against a specific allergen, a mechanism which may also be operative in the hyposensitisation therapy to bee venom allergens described previously (Kemeny et al., 1983a & b). T-cells are involved in the regulation of the humoral immune response (Gershon, 1974) and in the induction and maintenance of immunological tolerance (Katz and Benacerraf, 1974). Different populations of T-cells effect different regulatory functions; help, suppression and cytotoxicity (Cantor and Boyse, 1975). A possible use for highly purified and well characterised allergens is the development of modified derivatives which are capable of specifically inducing a suppressor T-cell response.

Experiments by Liu and colleagues with the major ragweed allergen, AgE, showed that immunisation with conjugates of AgE and a synthetic copolymer of D-glutamic acid and D-lysine, effectively a modified AgE, could specifically suppress the IgE response to native AgE (Liu and Katz, 1979). Suppression was persistent and antigen specific. Liu et al. (1979) subsequently demonstrated that suppression was mediated by an antigen-specific T-cell population. These observations agreed with the earlier work of Katz et al. (1974) who found that B-cells producing IgE were more susceptible to the regulatory influence of T-cells than were B-cells producing IgG. The AgE conjugate still retained at least some of its B-cell determinants, and, therefore, much of its native structure since it was recognised by anti-AgE antibodies. Conjugation, then, caused limited alteration to the protein but was still a crude tool since the exact sites and nature of the modifications were unknown. In

addition the fact that the modified allergen was recognised by B-cells would limit its use in immunotherapy as administration of even small doses could cause a severe allergic response in highly sensitised individuals. Similar experiments by Lee and Sehon (1981) demonstrated the induction of hapten-specific T-cells against polyvinyl alcohol conjugates of dinitrophenol.

These experiments demonstrate that it is possible to produce allergen derivatives with limited modifications which are capable of inducing an antigen specific, suppressor T-cell population. The future use of modified allergens in immunotherapy, then, may involve the production of specifically modified derivatives which are not only capable of inducing a suppressor T-cell population in the majority of individuals but which are not recognised by B-cells, that is, which do not elicit a hypersensitive response on administration. In this respect modified fragments of an allergen or the corresponding synthetic peptides should be of particular value since their small size will limit the number of possible B-cell determinants. *In vitro* mutagenesis of allergen cDNA clones may prove to be a useful tool in devising modified allergens with the desired biological activity.

The limitation to this approach is that each clinically important allergen would have to be modified and tested. In the absence of a predictive model describing the properties of T-cell determinants the choice of modifications would be essentially random.

A model of this type has recently been developed by DeLisi and Berzofsky (1985). They proposed that T-cell determinants are amphipathic, having a structure in which hydrophobic and hydrophilic residues occur on opposite faces, so that the hydrophilic face can interact with the T-cell receptor while the hydrophobic face interacts with the membrane of the accessory cell required in antigen presentation (for a review see Grey and Chestnut, 1985). Ideally the periodicity of

the hydrophobic and hydrophilic regions would coincide with that of a regular structure; for example a period of 3.6 residues consistent with an α-helix. They tested their hypothesis against twelve proposed T-cell sites from a range of proteins and found that it was valid for ten of them. Although they were unable to conclude that all amphipathic sites were T-cell determinants they could conclude that where a T-cell determinant occurs it will probably be amphipathic. If fully validated their results should simplify the search for T-cell determinants which would be restricted to amphipathic regions of the protein. This model is consistent with either of the opposing views on the nature of antigenic determinants described in Sub-section I 3(G) since it only delimits the regions of the protein which are likely to constitute T-cell determinants but does not predict which sites will be important in an individual response.

## Isotypic Regulation of the IgE Response

Although the approach to immunotherapy discussed in this section does not involve the use of purified allergens it will be described in order to give a full account of modern approaches to immunotherapy and to allow a more informed assessment of the potential role of allergens and their derivatives in this area.

Dissociation between the IgE and IgG responses is seen under a variety of experimental conditions (Levine and Vaz, 1970). The IgE response is dependant on the adjuvant used during immunisation; Bordetella pertussis vaccine and alum favour the IgE response while Complete Freund's Adjuvant has little effect except when administered repeatedly prior to injection of an antigen, in which case it suppresses the IgE response without affecting the IgG response. In both man and rats infestation with nematodes selectively enhances the IgE response to antigen subsequently administered. The dissociation between the IgG and IgE responses suggests that the synthesis of IgE is also regulated by some mechanism other than the antigen-specific T-cells described in the previous section. A third approach to immunotherapy is, therefore, possible; complete suppression of the IgE isotype. Capron et al. (1986) has noted that rare individuals lacking measureable serum or tissue IgE are apparently healthy implying that suppression of the IgE isotype will not have adverse side effects.

The experimental system that has been most fully characterised is that of rats infected with the nematode, Nippostrongylus brasiliensis. The results from this work have been comprehensively reviewed by Ishizaka (1984) and will be summarised here. T-cells expressing the Lyt1 surface marker ($Lyt1^+$) produce a 15 kDa polypeptide which binds IgE

and which either suppresses or potentiates the IgE response depending on its state of glycosylation. The IgE-suppressive factor has only O-linked oligosaccharides of which the terminal residue is galactose. The IgE-potentiating factor has both N-linked, mannose-rich oligosaccharides and O-linked oligosaccharides, the terminal residue of which is sialic acid (Yodoi et al., 1980; Yodoi et al., 1982). The balance between the IgE-potentiating and suppressive factors determines the strength of the IgE response.

For the selective formation of either IgE-potentiating or IgE-suppressive factors other soluble factors are required. Glycosylation-enhancing factor (GEF) is required for the formation of IgE-potentiating factor and glycosylation-inhibiting factor (GIF) is required for the formation of IgE-suppressive factor. GEF is produced by $Lyt1^+$ T-cells, has a molecular weight of 25,000 and an isoelectric point of 6.6 (Iwata et al., 1983a). GEF has lectin-like properties in that it can bind galactose or lactose residues. The IgE-binding activity of GEF can be inhibited by incubating T-cells with galactose suggesting that GEF exerts its effect at the level of the T-cells by first binding to surface galactose residues (Iwata et al., 1983b). GEF is also a serine protease related to kallikrein (Iwata et al., 1983b). It is known that kallikrein can liberate an activator of phospholipase, bradykinin, by cleavage of the precursor, kininogen (Pisano, 1974). Activation of phospholipase $A_2$ has been shown to enhance the glycosylation of IgE-binding protein (Yodoi et al., 1981). It is proposed that, in vivo, GEF cleaves kininogen or a related substance to release bradykinin or a related substance which stimulates glycosylation of IgE-binding factors by activating phospholipase $A_2$.

In contrast, GIF inhibits N-linked glycosylation of IgE-binding proteins. GIF has a molecular weight of 15,000-16,000 and is bound by an anti-lipomodulin antibody (Ueda et al., 1983). Lipomodulin, a

phospholipase inhibitory protein, purified from rat basophils has been shown to inhibit N-linked glycosylation of IgE-binding factors (Uede et al., 1983). GIF is probably lipomodulin or a related protein which inhibits N-linked glycosylation through its effect on phospholipase. Figure C1 summarises the current model for the isotypic regulation of IgE. Phospholipase $A_2$ has a central role in this model in controlling N-linked glycosylation of the IgE-binding factors. The mechanism by which phospholipase exerts this control is not known. Although these studies have been conducted in the rat similar IgE-binding factors have been demonstrated in human T-cell populations (Ishizaka and Sandberg, 1981; Huff and Ishizaka, 1984).

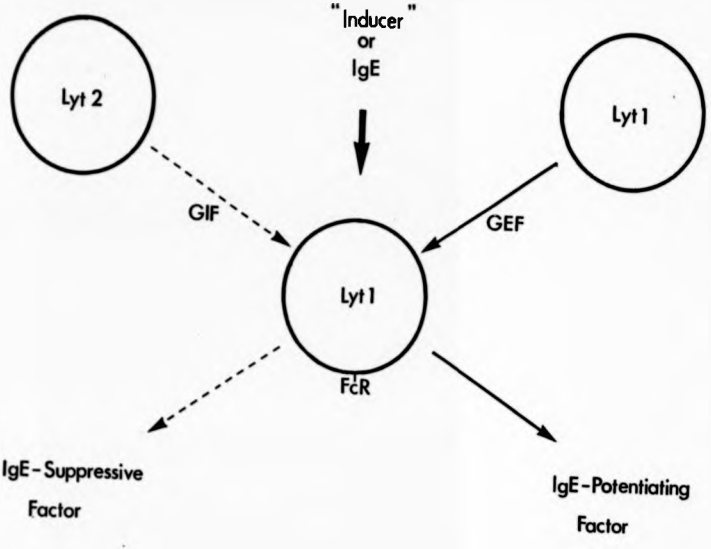
The existence of isotype-specific suppressor factors clearly provides a novel approach to the treatment of allergy. Shifting the balance of GEF and GIF in favour of GIF should suppress the IgE response. Intraperitoneal injection of GIF into mice was shown to reduce the IgE titre against an ovalbumin derivative, consistent with the model for IgE regulation. The IgE level was reduced to one eighth of the control value but the IgG response was also significantly reduced, to one half of the control value (Ishizaka et al., 1985). Administration of GIF in this way, then, does not result in isotype-specific suppression.

At this time none of the strategies outlined offers a comprehensive approach to the treatment or prevention of immediate hypersensitivity. It is likely that aspects of each will find practical application. Highly purified, well characterised allergens have a central role not simply in applied immunology such as immunotherapy but in the delineation of the antigenic structure of proteins and in the study of the genetic regulation of the immune response.

Figure C 1 Isotypic regulation of the IgE response

Figure C 1 is taken, with modifications, from the paper by Ishizaka et al. (1985) and summarises the model for the selective formation of either IgE-potentiating or IgE-suppressive factors.

GEF and GIF are abbreviations for glycosylation-enhancing factor and glycosylation-inhibiting factor, respectively. Lyt refers to a cell-surface marker used to distinguish between populations of T-cells. The model is discussed in Section C 4. FcR is a cell-surface receptor for immunoglobulin E heavy chain.


"Inducer"
or
IgE
Lyt 2
Lyt 1
GIF
GEF
Lyt 1
FcR
IgE-Suppressive
Factor
IgE-Potentiating
Factor

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